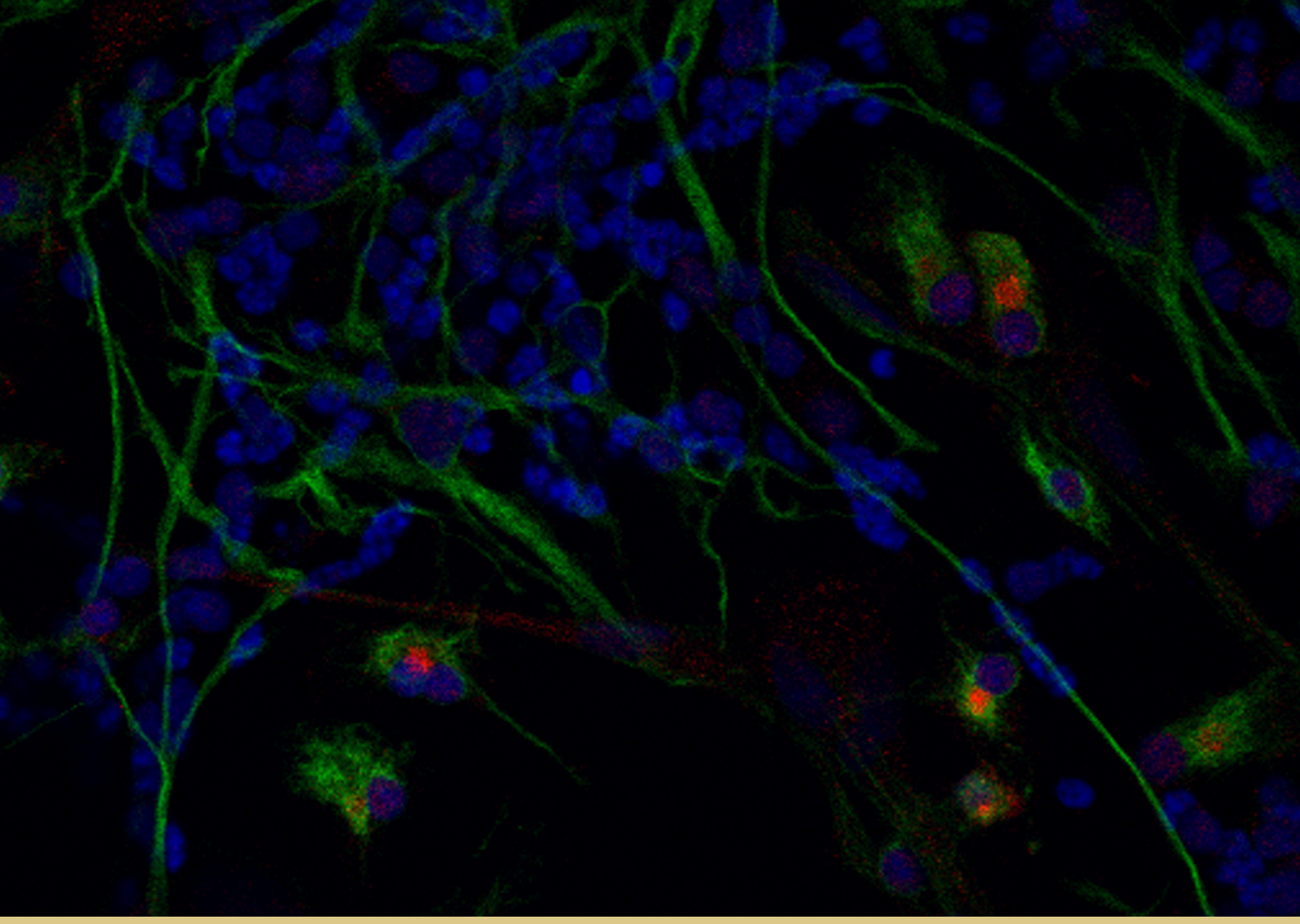

Joana Rita Domingues Vindeirinho

THE ADENOSINERGIC SYSTEM IN THE CONTEXT OF DIABETIC RETINOPATHY: FROM ALTERATIONS TO MODULATION

Tese de Doutoramento em Biologia Experimental e Biomedicina, Ramo de Neurociências e Doença orientada por Doutor Paulo Santos e Doutora Cláudia Cavadas e apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra (IIIUC)

Fevereiro de 2015

· U C ·

Universidade de Coimbra

Cover:

Image showing immunocytochemistry of primary retinal cell cultures. Müller cells and microglia appear in green (immunolabeling of vimentin), while immunolabeling for adenosine deaminase appears in red. Nuclei are stained with DAPI (blue).

INSTITUTO DE INVESTIGAÇÃO INTERDISCIPLINAR (III-UC)

The Adenosinergic System in the Context of Diabetic Retinopathy: From Alterations to Modulation

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Tese de Doutoramento em Biologia Experimental e Biomedicina, Ramo de Neurociências e Doença

Joana Rita Domingues Vindeirinho

Fevereiro 2015

Tese apresentada à Universidade de Coimbra (III-UC) para prestação de provas de Doutoramento em Biologia Experimental e Biomedicina, Ramo de Neurociências e Doença. Este trabalho foi realizado no Centro de Neurociências e Biologia Celular (CNC) e Institute for Biomedical Imaging and Life Sciences (IBILI) da Universidade de Coimbra sob orientação do Doutor Paulo Santos e Doutora Cláudia Cavadas. O trabalho foi efetuado ao abrigo de uma bolsa de doutoramento atribuída pelo programa doutoral em Biologia Experimental e Biomedicina do Centro de Neurociências e Biologia Celular da Universidade de Coimbra, financiada pela Fundação para a Ciência e Tecnologia (SFRH/BD/51195/2010).

(…)I am almost convinced (quite contrary to opinion I started with) that species are not (it is like confessing a murder) immutable.

- Charles Darwin
  In Correspondence to Hooker, J.D., 11 Jan,1844

É a tua palavra contra a minha imaginação.

- Ludgero Avelar

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Abbreviations

5’-NT	5’-ecto-nucleotisade
A_1R	A_1 adenosine receptor
$A_{2A}R$	A_{2A} adenosine receptor
$A_{2B}R$	A_{2B} adenosine receptor
A_3R	A_3 adenosine receptor
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AGA	Amadori-glycated albumin
AGE	Advanced glycation end-product
AK	Adenosine kinase
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BRB	Blood retinal barrier
BSA	Bovine serum albumin
CBP	CREB-binding protein
CGS21680	4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid
CLAP	Chymostatin, pepstatin, antipain and leupeptin
CMF	Ca^{2+} and Mg^{2+}-free Hank´s balanced salt solution
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
Co-IP	Co-immunoprecipitation
CREB	cAMP-response element-binding protein

Abbreviations

DMSO	Dimethyl sulfoxide
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DPP4	Dipeptidyl peptidase 4
DR	Diabetic retinopathy
ECF	Enhanced chemifluorescense substrate
ENT	Equilibrative nucleoside transporter
ERG	Electroretinogram
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FELASA	European Federation for Laboratory Animal Research
FFT	Fast fourier transform
GABA	γ-Aminobutyric acid
GCL	Ganglion cell layer
GDM	Gestational diabetes
GPCR	G protein coupled receptors
HG	High glucose
HPLC	Reverse-phase high-performance liquid chromatography
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMP	Inosine monophosphate
INL	Inner nuclear layer
i.p.	Intraperitoneal
IP_3	Inositol 1,4,5-triphosphate
IPL	Inner plexiform layer
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium

NBMPR	S6-(4-nitrobenzyl)-mercaptopurine riboside
NTPDases	Ectonucleoside triphosphate diphosphohydrolases
NFκB	Nuclear factor κB
NFL	Nerve fiber layer
NO	Nitric oxide
NPY	Neuropeptide Y
OCT	Optimal cutting temperature compound
ONL	Outer nuclear layer
OP	Oscillatory potential
OPL	Outer plexiform layer
PBS	Phosphate buffer saline
Pi	Inorganic phosphate
PI	Propidium iodide
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PL	Photoreceptor layer
PLC	Phospholipase C
PMSF	Phenylmethanesulphonylfluoride
Ppia	Peptidylprolyl isomerase A
PVDF	Polyvinylidene difluoride
qRT-PCR	Real-time quantitative polymerase chain reaction
RAS	Renin angiotensin system
RGC	Retinal ganglion cell
R_{max}	Asymptotic maximum response
RNApol2	RNA polymerase 2
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium

Abbreviations

SAH	S-adenosylhomocysteine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

Resumo

A retinopatia diabética é uma das complicações mais comuns da diabetes e a principal causa de cegueira em adultos. Os estádios iniciais da retinopatia diabética exibem características típicas de um ambiente excitotóxico e de inflamação crónica, como a libertação excessiva de glutamato, a ativação de microglias e a libertação de mediadores inflamatórios. A adenosina é um nucleósido de purina que é libertado em condições adversas e que pode ativar quatro tipos de recetores de adenosina (A_1R, $A_{2A}R$, $A_{2B}R$ e A_3R). Todos estes recetores se encontram presentes na retina e a sua ativação regula tanto mecanismos de neurotransmissão, como processos inflamatórios. Estudos mostram que vários componentes do sistema adenosinérgico são afetados por condições diabéticas em vários tecidos e modelos animais mas, mesmo com algum progresso feito, a influência que as condições diabéticas exercem neste sistema na retina ainda é pouco compreendida. Devido a isto, um dos objetivos deste trabalho foi o de esclarecer os efeitos que a diabetes causa ao sistema adenosinérgico na retina.

Usámos culturas primárias de células da retina expostas a glicose elevada, para mimetizar condições de hiperglicemia característica da diabetes, e ratos tornados diabéticos por aplicação de estreptozotocina (modelo de diabetes tipo 1), como modelos para estudar o efeito que estas condições exercem nos níveis de expressão de recetores de adenosina, de transportadores de nucleósidos e de enzimas chave na remoção de adenosina como ADA e AK. Para além disso, avaliámos os níveis de atividade das NTPDases e da 5'-NT, enzimas responsáveis pela cadeia de degradação de purinas, e também os níveis de adenosina em condições de alta glicose. Também explorámos uma ligação entre ADA e DPP4 quando a glicose se encontra elevada.

Resumo

Demonstrámos que a expressão dos recetores A_1R, $A_{2A}R$ e A_3R está alterada em células da retina quando expostas a elevada concentração de glicose, e também em retinas de ratos diabéticos. O transportador de nucleósidos equilibrativo 1 tem expressão aumentada em condições de alta glicose, enquanto que o transportador concentrativo 2 está elevado em retinas após quatro semanas de diabetes. As enzimas de remoção de adenosina apresentam expressão diminuída em retinas após quatro semanas de diabetes, ao passo que a atividade das NTPDases aumenta com glicose elevada, assim como aumentam os níveis de adenosina extracelular. De igual modo demonstrámos que DPP4 é capaz de influenciar não só a atividade da ADA, como os níveis proteicos desta enzima, um efeito apenas parcialmente bloqueado por um inibidor da atividade enzimática da DPP4.

Uma vez que o ambiente característico dos estádios iniciais da retinopatia diabética inclui várias condições moduláveis pela sinalização por adenosina, como excitotoxicidade, inflamação e angiogénese, é provável que os diferentes recetores de adenosina possam ser um potencial alvo terapêutico para o tratamento da retinopatia diabética. Devido a isto, procurámos avaliar os efeitos que a modulação do sistema adenosinérgico poderá ter na morte celular que acompanha as condições de alta glicose, e contra a neurodegeneração e perda de função que anuncia a retinopatia diabética.

Avaliámos a modulação da atividade dos A_1R e $A_{2A}R$, assim como o bloqueio do ENT1, em culturas de células de retina sujeitas a glicose elevada. Para além disso, um antagonista do $A_{2A}R$ foi administrado a ratos e o desempenho da retina avaliado por eletrorretinografia, os níveis de morte neuronal avaliados por quantificação da perda de células ganglionares da retina, e as condições de inflamação avaliadas através da medição dos níveis de expressão das citocinas pró-inflamatórias TNF e IL-1β.

Observámos que a ativação do A_1R foi capaz de prevenir o aumento de morte celular induzido por glicose elevada, e que o bloqueio do $A_{2A}R$, assim como do transporte por ENT1, provou ser igualmente eficaz na prevenção da morte celular nas mesmas

condições. Para além disso, um tratamento de sete dias em ratos diabéticos com um antagonista do $A_{2A}R$ demonstrou um potencial efeito benéfico contra a disfunção da retina causada por diabetes, com melhorias observadas na amplitude da onda b escotópica e na latência dos potenciais oscilatórios. O bloqueio do $A_{2A}R$ foi igualmente benéfico contra morte neuronal, ao melhorar os níveis de sobrevivência das células ganglionares da retina em ratos diabéticos tratados.

Em resumo, este trabalho mostra que o sistema adenosinérgico da retina sofre alterações na maioria dos seus componentes em condições de alta glicose e nas retinas de ratos diabéticos. De igual modo demonstrámos que a modulação da sinalização por adenosina melhora a sobrevivência de células da retina em situações de glicose elevada ou de diabetes, prevenindo também algumas respostas eletrofisiológicas anormais observadas nas retinas diabéticas, sugerindo assim que a modulação da atividade do $A_{2A}R$ poderá constituir uma nova estratégia farmacológica no tratamento da retinopatia diabética nos seus estádios iniciais.

Abstract

Diabetic retinopathy is one of the most common complications of diabetes and the leading cause of blindness in working age adults. The early stages of diabetic retinopathy exhibit characteristics of an excitotoxic environment and chronic inflammation, such as excessive glutamate release, microglia activation and release and accumulation of pro-inflammatory mediators. Adenosine is a purine nucleoside that can be released under adverse conditions, and can activate four types of adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R). All four receptors are present in the retina, and its activation regulates both mechanisms of neurotransmission and inflammatory processes. Previous studies using different models have shown that several components of the adenosinergic system are affected by diabetic conditions in several tissues but, despite some progress made, the influence that diabetic conditions exert on this system in the retina is still poorly understood. Because of this, a major objective of this work was to uncover the effects of diabetes on the adenosinergic system in the retina.

We used primary cultures of mixed retinal cells exposed to elevated glucose, to mimic hyperglycemic conditions, and streptozotocin-induced diabetes (one and four weeks duration) in Wistar rats (type 1 diabetes model), two models used to study the effect exerted by hyperglycemic conditions on the expression levels of adenosine receptors, nucleoside transporters, and of key metabolizing enzymes for the removal of adenosine, such as adenosine deaminase (ADA) and adenosine kinase. We also evaluated the activity levels of NTPDases and of 5'-NT, enzymes responsible for the degradation of ATP, ADP and AMP, as well as the total levels of adenosine under high glucose conditions. We also explored the interaction and influence that DPP4 has on ADA when glucose is elevated.

Abstract

We have shown that the expression of adenosine receptors A_1R and $A_{2A}R$, as well as the equilibrative nucleoside transporter 1, are altered in retinal cells exposed to high glucose. Elevated glucose increased the activity of NTPDases, along with extracellular adenosine levels. We also found that DPP4 is able to modulate ADA activity and also affect ADA protein levels, an effect that was only partially abolished by a DPP4 inhibitor.

In diabetic retinas at one and four weeks of diabetes, the expression of A_1R, $A_{2A}R$ and A_3R is altered, while the concentrative transporter 2 increased, and the adenosine removing enzymes (ADA and AK) decreased after four weeks of diabetes. Elevated glucose also increased the activity of NTPDases, along with the extracellular adenosine levels. We also found that DPP4 is able to modulate ADA activity and also affect ADA protein levels, an effect that was only partially abolished by a DPP4 inhibitor.

Because several conditions characteristic of the microenvironment of the early stages of diabetic retinopathy are affected by adenosine signaling, such as excitotoxicity and inflammation, it is conceivable to consider adenosine receptors as potential therapeutic targets. Because of this, we evaluated how modulation of the adenosinergic system could be protective against retinal cell death triggered by exposure to elevated glucose and against neurodegeneration and impaired retinal physiology in the diabetic retina.

We assessed the modulation of A_1R and $A_{2A}R$ activity, as well as the inhibition of ENT1 transport, on retinal cell cultures exposed to high glucose. Beyond that, an $A_{2A}R$ antagonist, SCH 58261, was administered to control and diabetic rats, and the retinal function assessed by performing ERG recordings. Retinal ganglion cell loss was quantified and inflammatory processes studied, by evaluating the expression of pro-inflammatory cytokines, TNF and IL-1β.

Activation of A_1R was able to prevent the increase in cell death caused by exposure to elevated glucose. Blockade of $A_{2A}R$ and ENT1 transport was also very effective in preventing cell death under the same experimental conditions. As for the studies in

diabetic rats, treatment with the $A_{2A}R$ antagonist for seven days exerted a potential beneficial effect on retinal dysfunction caused by diabetes, with improvements detected in scotopic b-wave amplitude and latency of oscillatory potentials. $A_{2A}R$ blockade was also beneficial against neuronal cell death, improving retinal ganglion cell survival in diabetic rats.

In conclusion, this work shows that in retinal cells exposed to hyperglycemic conditions and in the diabetic retina, the adenosinergic system undergoes alterations in the majority of its components. Furthermore, we have shown that modulation of adenosine signaling can improve retinal cell survival in both cell cultures exposed to elevated glucose and in diabetic retinas, preventing also some retinal electrophysiological abnormalities detected in diabetic retinas, suggesting that modulation of $A_{2A}R$ may constitute a potential new therapeutic strategy to treat diabetic retinopathy in its early stages.

Chapter 1 - Introduction

1.1 The Retina

The retina lines the inner, posterior surface of the eye, and is a part of the central nervous system (CNS) due to being developed from outpocketings of the neural tube. It is characterized by a defined, layered structure comprised of three cell layers separated by two additional layers that contain almost no cell nuclei. The retina contains several types of cells: neuronal cells (1st, 2nd, and 3rd order), macroglial and microglial cells, blood vessel cells and pigmented epithelial cells (Gardner et al., 2002). As illustrated in Figure 1, the outermost layer of the retina is the retinal pigment epithelium (RPE), a monolayer consisting of cells with high melanin content, the retinal pigmented epithelial cells, that have several roles from increasing the absorption of scattered light and maintenance of the blood-retinal barrier (BRB), to the recycling of photopigments and phagocytosis of the outer segments of photoreceptors. These photoreceptors border the RPE and form the two next layers: The outer segments of these cells, containing membranous disks storing light sensitive visual pigments, form the photoreceptor layer (PL) while the inner segments, rich in mitochondria, and cell body form the densely packed outer nuclear layer (ONL). Adjacent to the ONL is the outer plexiform layer (OPL), where photoreceptors (1st order neurons) form synapses with bipolar cells and horizontal cells, both 2nd order neurons. The cell bodies of these neurons, along with the nuclei of amacrine cells (3rd order neurons) and Müller cells, form the inner nuclear layer (INL), and both bipolar and amacrine cells form synapses with the retinal ganglion cells in the inner plexiform layer (IPL) (“Simple Anatomy of the Retina by Helga Kolb – Webvision,” n.d.). The cell bodies of retinal ganglion cells and some amacrine cells form the next layer, the ganglion cell layer (GCL), while the ganglion cell axons, responsible for relaying information to the brain visual centers, spread across the retina surface forming the nerve fiber layer (NFL) before coming to the optic disk and are packed together, forming the optic nerve.

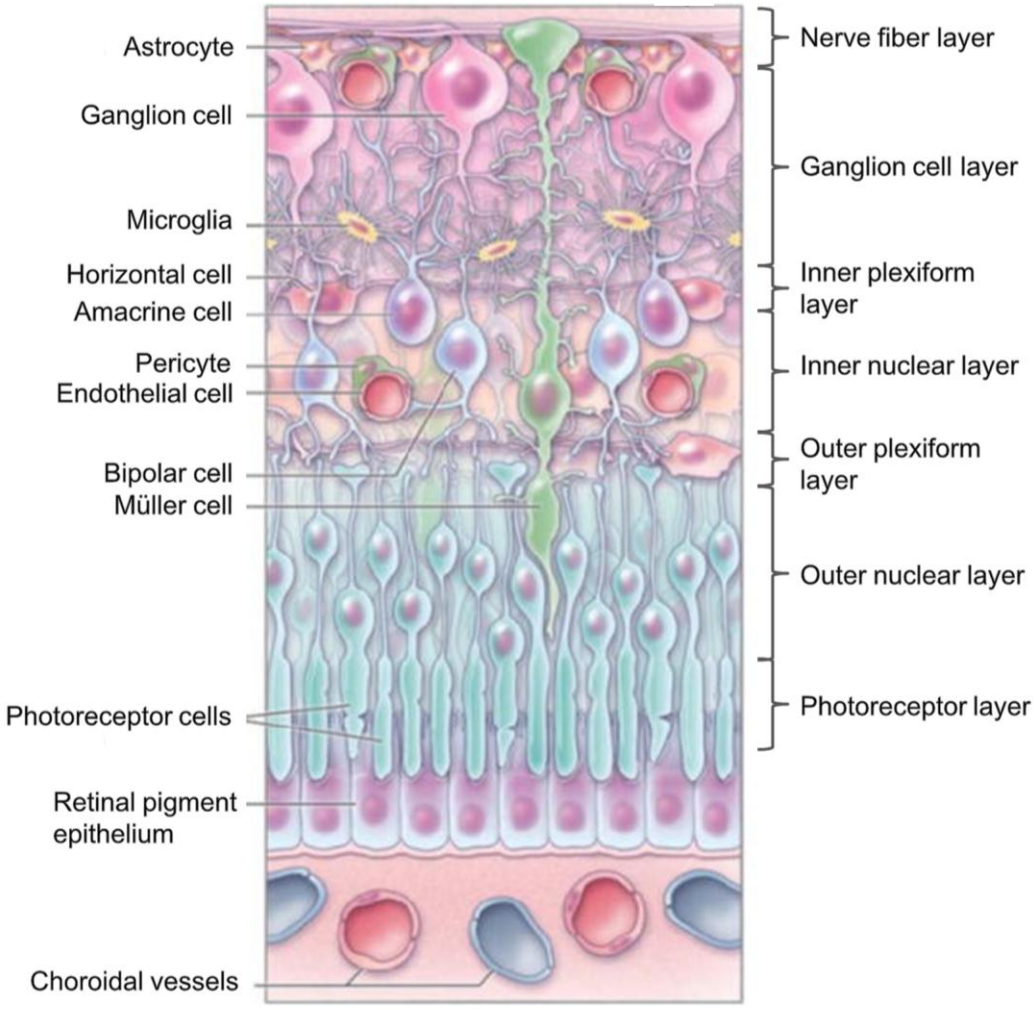

Figure 1: Schematic representation of a retinal cross section detailing the different layers and cell types. (adapted from (Stem and Gardner, 2013), with permission)

Due to the position and orientation of the retinal layers, any light entering the eye must travel through not only blood vessels, but most of the retinal layers before it reaches the photoreceptors, leading to an increase in scattered light (reducing visual acuity) and the creation of a blind spot, where the optic disk is located (FRSE et al., 2008). While the blind spot is inevitable, the light scattering was compensated by the development of the fovea (*fovea centralis*), a specialized region found in primates and some non-mammalian vertebrates, such as birds and reptiles ((*Encyclopedia of Neuroscience* | *Springer*, n.d.). The fovea is a depression at the surface of the retina that forms in an avascular region of the central macula due to mechanical forces during

development (Provis et al., 2013). The photoreceptors in this region are almost entirely cones, the photoreceptors responsible for chromatic and acute vision that are elongated and densely packed. Ganglion cell density is also increased in the fovea, but the depression of the fovea means that the cells of the inner retina are moved aside and this, along with the absence of blood vessels allows a much clearer path for the light to reach the photoreceptors (Provis et al., 2013) and reduces light scattering.

1.1.1 Retinal Neurons

The population of neuronal cells in the retina is very complex and diverse, with some authors proposing over 60 different types, each with defined roles in the processing of visual information (Masland, 2012). Nonetheless, they can be grouped into five categories: photoreceptors, bipolar, horizontal, amacrine, and ganglion cells. As described previously, photoreceptors are considered the 1st order neurons, bipolar and horizontal cells are the 2nd order neurons and amacrine and retinal ganglion cells the 3rd order neurons.

Photoreceptors, due to their photosensitive pigments, are the neurons crucial for the process of phototransduction, which translates light into an electrochemical signal. These photopigments consist of a g-protein coupled receptor (GPCR), opsin, bound to an organic chromophore. In mammals, the chromophore is 11-*cis*-retinal (Wald, 1968), a vitamin A-based retinaldehyde, and is the key component of the photopigment function. As for the protein, there are several types of opsins, with an average size of 30-60 kDa, that envelop the chromophore and modulate the environment, maximizing the absorption of light of particular wavelengths for each opsin, therefore providing a method for the differential sensing of diverse wavelengths and allowing for color vision (Shichida and Matsuyama, 2009). Upon light absorption, the retinal changes conformation from 11-*cis* to all-*trans* and, due to the tight binding to the opsin, this

causes structural changes in the opsin that activate the G-protein coupled to it (Shichida and Matsuyama, 2009).

The different opsins can be separated into two general groups, according to their allocation to two distinct photoreceptor types: rod opsin, or rhodopsin, present in rod photoreceptors, and cone opsins, present in cone photoreceptors. Rod photoreceptors are narrow and elongated, with slow action, low acuity and resolution but high sensitivity in scotopic conditions, while cone photoreceptors have shorter outer segments and have a lower sensitivity but fast action, high acuity and resolution in photopic conditions (Fain et al., 2010). Both rod and cone photoreceptors respond to light by hyperpolarizing and while rods contain only rhodopsin, cones are further divided by the cone opsin they contain. In the human retina we find cones that contain opsins sensitive to light of short (blue), medium (green), or long wavelength (red) (Shichida and Imai, 1998). Despite the higher diversity of cones, rods comprise the vast majority of photoreceptors in the human retina, with around 90 million of the 94.5 million photoreceptors present (Purves et al., 2011). Cones however, are in higher density in specific areas, such as the macula and fovea, as mentioned previously, providing detailed spatial resolution and fine detail perception (Kolb et al., 2001; Provis et al., 2013). After the visual stimuli, visual information is transmitted from photoreceptors to the bipolar cells, when light stimuli reduce the release of glutamate from photoreceptors.

Several types of bipolar cells can be described based on morphology, function and distribution, with the highest number proposed being 12 types (Masland, 2012), but they are generally grouped into four broad types according to the expression of different glutamate receptors and therefore their response to glutamate release. OFF bipolar cells express α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors leading to the cell being hyperpolarized in light conditions. Conversely, ON bipolar cells express mGluR6, a metabotropic receptor that, when activated by glutamate, closes the cation channel TRPM1 which means that, in light

conditions these cells depolarize (Masland, 2012). Bipolar cells can also be sustained or transient, according to the speed of the inactivation of glutamate receptors, which leads to the four broad types of bipolar cells: ON-transient, ON-sustained, OFF-transient or OFF-sustained. Bipolar cells form synapses exclusively with either rods or cones, and can make connections with one photoreceptor only or many at the same time (Masland, 2012).

In the IPL, the short axons of bipolar cells transmit the visual information through synapses with the retinal ganglion cells. Glutamate is again the main neurotransmitter, while other neurotransmitters released by amacrine cells modulate the signaling cascade (Daw et al., 1989). Ganglion cells are also very diverse, with around 10-20 types (Rockhill et al., 2002), with distinct morphologies, physiologies, and molecular signatures, and these allow ganglion cells to receive information from just one or several bipolar cell types, and incorporate visual inputs that will transmit different representations of visual stimuli to the brain (Masland, 2012). This system allows for a convergence of information at the ganglion cell level, a fact that is evident in the disparity between photoreceptor and ganglion cell numbers (around 100 million to 1.6 million respectively) (Hall, 2010). This convergence is not constant throughout the retina, however, and regions with higher visual acuity have numbers close to one photoreceptor per ganglion cell (FRSE et al., 2008).

An important regulation of the signaling cascade of visual information is performed by the horizontal cells and amacrine cells, lateral interneurons located in the ONL and INL, respectively. Horizontal cells are spread laterally, connecting with neighboring horizontal cells by gap-junctions, and performing their modulatory functions in the OPL. They receive input through the release of glutamate by photoreceptors and then provide inhibitory feedback to them, as well as further signaling to bipolar cells, by releasing γ-Aminobutyric acid (GABA) (Cueva et al., 2002; Masland, 2012). Horizontal cells are important for local gain control of the retina, allowing objects of different levels

of brightness to be detected in the same visual field, and they can also be involved in the enhancement of object edges, or “center-surround” organization (Masland, 2012).
As for amacrine cells, these interneurons have high structural diversity, with some cells (wide-field amacrine cells) spanning large distances in the retina, and establish multiple connections at the IPL, receiving input and establishing feedback mechanisms with bipolar cells, while also connecting with ganglion cells and with other amacrine cells (Masland, 2012). Like horizontal cells, amacrine cells also use GABA as the main neurotransmitter, although glycine and acetylcholine are also released in some types of amacrine cells (Santos et al., 1998). They are responsible for processing information in numerous visual tasks and offer numerous contextual effects that modulate ganglion cell response, such as involvement in the “center-surround” organization, object motion detection (separating true object motion from self-induced motions such as eye movements) (Gollisch and Meister, 2010), and directional sensitivity (Yoshida et al., 2001).

1.1.2 Retinal macro and microglia

The retinal glial cells are heavily involved in the maintenance of retinal function and internal stability, offering structural support, regulating metabolism, modulating the function of neurons and vascular cells, and also immune surveillance and action. Three types of glia can be found in the retina: astrocytes, Müller cells and microglia. Unlike the other regions of the CNS, retinal axons are not myelinated which means there are no oligodendrocytes in the retina, and they only appear in the optic nerve (Ffrench-Constant et al., 1988; Perry and Lund, 1990).
Astrocytes and Müller cells are commonly referred to as retinal macroglia and, despite clear morphological differences and separate retinal origins (Watanabe and Raff, 1988; Turner et al., 1990), these cell types share numerous functions: both are vital for the integration of vascular and neuronal activity in the retina by enveloping blood vessels,

regulating metabolic support for neurons, and maintaining the BRB. Müller cells are the main glial cells in the retina, and are only found in this tissue, developing from the same progenitor cells that form retinal neurons (Turner et al., 1990). They are specialized radial cells found in the INL, although their processes cross the thickness of the retina contacting all neuronal processes (Bringmann et al., 2006). Starting from retinal development on, Müller cells are crucial in creating and maintaining neuronal architecture, and supporting neuronal survival and proper function: They regulate glucose metabolism and retinal blood flow, maintain ion and water homeostasis and are also involved in neuronal signaling processes (Bringmann et al., 2006). Due to their distinct energy metabolism, Müller cells are able to survive in severe conditions such as anoxia, ischemia or hypoglycemia, and have the ability to switch to a reactive state in response to pathological conditions, called Müller cell gliosis, allowing these cells to becoming key players in these situations. Once activated, Müller cells can become neuroprotective in a number of ways, including buffering elevated potassium levels, uptake of excessive glutamate, and the release of numerous neurotrophic factors, growth factors, and cytokines, among others (Bringmann et al., 2009). However, Müller cell gliosis can also be detrimental: the downregulation of proteins involved in specific Müller cell functions during gliosis causes the disruption of normal glio-neuronal interactions and homeostasis, exacerbating damage and degeneration; Activated Müller cells can also release pro-inflammatory cytokines and produce excessive levels of nitric oxide (NO). Often, the same response can be benefic or damaging, depending on time and amplitude (Bringmann et al., 2009).

Astrocytes and their processes are more restricted than Müller cells, being found in the GCL and NFL, and interact with vascular cells and ganglion cells. These cells do not develop in the retina itself, but immigrate from the optic nerve. Astrocytes are only present in vascular regions (Schnitzer, 1987) of the retina where, like Müller cells, they are able to induce the formation of tight junctions between epithelial cells by releasing

factors (Janzer and Raff, 1987), highlighting their role in the development and maintenance of retinal vasculature.

Apart from the macroglia, the retina also possesses microglia, the resident immune cells of the CNS. Like astrocytes, microglia are not native to the retina, developing from mesodermal/mesenchymal progenitors and migrating to the retina at the embryonic and fetal stages of development (Chan et al., 2007). Once there, they undergo morphological changes and adapt to the local microenvironment (Chen et al., 2002). During development, microglia are associated with control of developmental apoptosis, clearance of debris, the regulation of synaptogenesis and shaping of neuronal connections (Karlstetter et al., 2014). In the adult retina, microglia can be primarily found in the GCL and both plexiform layers, where they spread highly ramified processes that occasionally cross nuclear layers (Zhang et al., 2005) while in a quiescent state, forming a network that is able to constantly monitor the entire retina (Nimmerjahn et al., 2005), and allows microglia to engage with neurons and macroglia. Because of this sensitivity to alterations in the retina, microglia are closely controlled by other retinal cells in a healthy retina by release of inhibitory factors and binding of transmembranar glycoproteins (Karlstetter et al., 2014). However, when a response is elicited by injury or inflammation, microglia switch to an activated state, and undergo morphological changes, retracting their processes and assuming an amoeboid appearance (Streit et al., 1999). Once active, microglia can migrate to a site of injury by following chemotactic signals (Davalos et al., 2005; Karlstetter et al., 2014), increase production and release of inflammatory cytokines (Hanisch, 2002), and perform scavenging functions such as phagocytosis of dead cells and other debris (Petersen and Dailey, 2004). Due to the important role microglia play in sensing alterations to homeostasis and leading the retinal inflammatory response, microglia activation is a hallmark of many retinal diseases, from human retinitis pigmentosa and age-related macular degeneration, to glaucoma and diabetic retinopathy (Karlstetter et al., 2014).

1.1.3 Retinal vasculature

Due to the nature of retinal function, that requires high energy expenditure (Laughlin et al., 1998), and retinal morphology with regions adapted for high levels of visual acuity (*Encyclopedia of Neuroscience* | *Springer*, n.d.), the retina is a very demanding tissue to provide with the appropriate blood supply. To achieve this, the primate retina is irrigated by two circulatory systems, the choroidal and the retinal vasculature. The choroid stands between the sclera and the RPE, is highly vascularized, and provides the necessary oxygen and nutrients to avascular areas that comprise the highly metabolic photoreceptors, the RPE, and even the OPL, by a process of diffusion (Yu and Cringle, 2001). Choroid capillaries (choriocapillaris) possess perforations in the endothelial wall called fenestrae, to facilitate the movement of water and solutes to the extracapillar space facing the RPE (Nagy and Ogden, 1990). Also helping this task is the high flow rate of the choroid, representing more than 65% of the blood flow to the retina (Records, 1979). The remaining blood flow is then supplied by the retinal vasculature, which irrigates the inner retina, including the IPL, counted as one of the highest oxygen consuming layers of the retina, together with the photoreceptor layers (Yu and Cringle, 2001). Here there are no fenestrations, and endothelial cells bind together by tight junctions, creating the foundation of the inner BRB, restricting movement of ions and large molecules to and from the retina, a structure essential for the conservation of retinal homeostasis (Cunha-Vaz et al., 1966) and the preservation of an environment free from most immunological influences (Engelhardt, 2006). Because of the choroidal fenestrations, the outer BRB is not maintained by the corresponding choroid vessels, but by the epithelial cells of the RPE (Strauss, 2005).

The main cell types that shape the choroid and retinal vasculature are the endothelial cells, forming the inner lining of vessels, and the pericytes, which lie outside the capillary and ensheat the endothelium. Endothelial cells, as mentioned before, are vital

for the maintenance of the BRB, while pericytes control endothelial cell permeability (Armulik et al., 2010), and regulate microvascular blood flow (Bandopadhyay et al., 2001).

1.2 Diabetes mellitus

Diabetes mellitus, commonly referred by diabetes, is a group of chronic diseases characterized by elevated blood glucose levels (hyperglycemia), and is one of the most widespread non-communicable diseases in the world. Currently, over 387 million people are estimated to live with diabetes (Figure 2), a number that is rising faster than predicted, due to its close relation with development of nations, urbanization, and changes in lifestyle (“Diabetes,” 2014). In Portugal, over one million of the adult population (29-79 years) is estimated to live with diabetes in 2013, comprising 13% of a total of 7.8 million people (“Direcção-Geral da Saúde,” 2014), a number aggravated by the demographic shift toward an aged population. The heavy financial burden caused by diabetes can be illustrated by the fact that in 2013 alone, the Portuguese healthcare system spent an estimate of 1250 to 1500 million Euros on diabetes-related healthcare (“Direcção-Geral da Saúde,” 2014).

There are three main types of diabetes: Type 1 (T1DM), type 2 (T2DM) and gestational diabetes (GDM). T1DM is the most severe form, but not the most common, representing less than 10% of all diabetic cases. It is caused by destruction of pancreatic β cells, with the concomitant decrease in the production and secretion of insulin, leaving the body unable to adapt to shifts in metabolic needs. This irreversible damage is caused, in most cases, by an autoimmune reaction and because of this, T1DM progresses quickly from the onset.

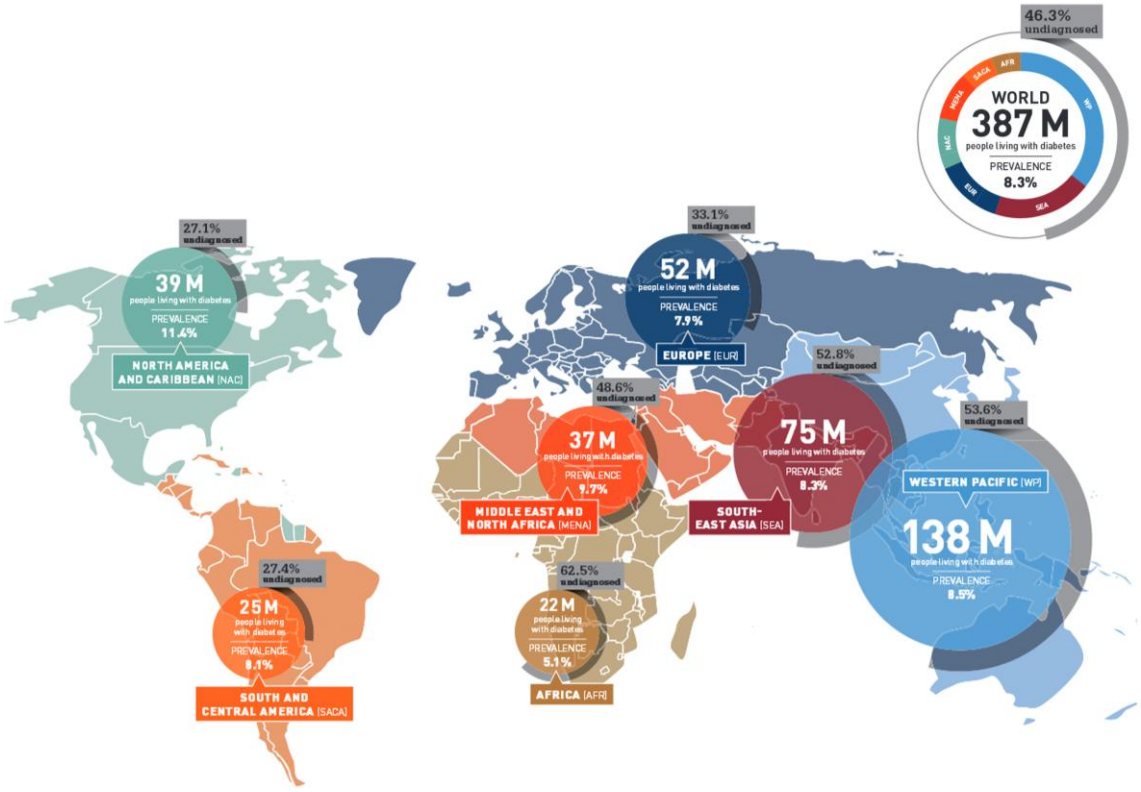

Figure 2: Diabetes Atlas for 2014. Estimate number of diabetic patients per world region. (adapted from IDF Diabetes Atlas, sixth edition, 2014 update, ("Diabetes," 2014), free use)

Conversely, T2DM has a slow progression and is commonly undiagnosed for several years after the onset of the disease. It is characterized by a chronic insulin resistance, and sometimes accompanied by a relative decrease in insulin secretion. Obesity, unhealthy diets, and a sedentary lifestyle are risk factors for T2DM, along with a strong hereditary component. It is the most common form of the disease, constituting 85-90% of diabetes cases. Having a slow progression, T2DM is generally less severe than T1DM, although in advance stages patients may develop insulin deficiency (Turner et al., 1997).

GDM corresponds not to a disease *per se* (Buekens, 2000), but refers to abnormal plasma glucose and/or oral glucose tolerance test values that occur during pregnancy in non-diabetic women and as such, is the only diabetes type that is not a chronic condition. Hormonal alterations and other factors are known to interfere with proper

insulin receptor signaling, and some degree of insulin resistance is a common outcome emerging in the second trimester of pregnancy. In GDM cases, however, these alterations are aggravated to levels similar to T2DM, and the precise mechanisms underlying this are still unknown, although the prevailing theory is that GDM arises from the inability of the pancreatic β cells to compensate the pregnancy-related insulin resistance. If untreated, GDM poses immediate risks to mother and fetus, but also increases the probability of future complications such as metabolic syndrome, T2DM and cardiovascular diseases (Nolan, 2011).

After the breakthrough of insulin administration as a viable treatment for diabetes, premature death decreased significantly and quality of life for diabetic patients increased dramatically (Rosenfeld, 2002). Although still incurable, diabetes became a manageable chronic disease, which led to the rise in the incidence of diabetes-related complications.

1.2.1 Diabetic Retinopathy

As mentioned before, the progression of diabetes carries with it several diabetes-related complications, affecting several organs and mechanisms, such as heart, kidneys, the eye, and the process of wound healing (“The Diabetes Control and Complications Trial Research Group,” 1993; Group, 1995). Even with good glycaemic control, there is increased risk of developing these conditions with age and duration of diabetes.

Diabetic retinopathy (DR) is the most common complications of diabetes (Simó et al., 2014). It is highly prevalent in patients with T1DM, with a global estimate of 77.3% of patients presenting some form of DR (Yau et al., 2012), and over 60% of those with T2DM have some degree of retinopathy after having had the condition for 20 years (“Diabetes,” 2014), making it the leading cause of visual impairment and preventable blindness in working age adults (Cheung et al., 2010; Yau et al., 2012).

1.2.1.1 Pathology of DR

DR has classically been regarded as a disease of the retinal microvasculature, starting with a progressive permeabilization of the BRB, vascular occlusion, formation of macular edema and tissue ischemia. DR can then evolve to a more damaging stage, with proliferation of new blood vessels, increased ischemia and retinal detachment (Cunha-Vaz, 2007).

The progression of DR has been subjected to several classification methods, with the proposed Clinical Diabetic Retinopathy Disease Severity Scale being a simple and clinically suitable classification (Wilkinson et al., 2003) into five stages of increasing severity, each defined by the clinical signs found during eye fundus examination (Figure 3):

1) No apparent retinopathy
2) Mild non-proliferative DR
3) Moderate non-proliferative DR
4) Severe non-proliferative DR
5) Proliferative DR

The first of the non-proliferative stages is diagnosed when microvascular lesions are detected in fundus examination, usually microaneurysms resulting from dilation of capillary walls (Cunha-Vaz, 1978). As the damage progresses, other signs become apparent upon examination, such as intraretinal hemorrhages in the middle layers of the retina, hard exudates and lipid deposits formed upon BRB impairment. These signs indicate the levels of vascular permeability which, as the disease progresses, causes the formation of macular edema by the accumulation of fluids that can lead to severe visual loss if untreated. In the most advanced non-proliferative stage, capillary

occlusions and fine irregular vessels are present in examinations, as well as the dilation of major retinal vessels in association with ischemic regions. The evidence of neovascularization marks the final, proliferative stage, where the hypoperfusion caused by capillary occlusion induces the formation of new blood vessels, mainly through the release of vascular endothelial growth factor (VEGF) by cells under hypoxic conditions (Adamis et al., 1994; Miller et al., 1994), most likely in an attempt to revascularize the ischemic tissue. While loss of visual acuity in DR results mainly from macular edema (Moss et al., 1988), the resulting ischemia, and the intraretinal and vitreous hemorrhages can also impair central vision, while leaving peripheral vision unaffected. In the more advanced cases of DR, the retinal detachment caused by extensive neovascularization can result in severe, irreversible vision loss.

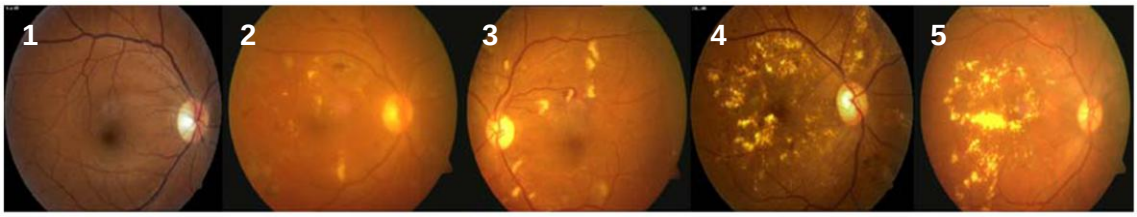

Figure 3: Representation of the stages of diabetic retinopathy. 1) normal retina; 2) mild non-proliferative DR; 3) moderate non-proliferative DR; 4) severe non-proliferative DR; 5) proliferative DR. (in Nayak et al., 2008, with permission)

1.2.1.2 DR as a neurovascular disease

As evidenced by the extensive damage that microvascular alterations can cause if untreated in DR and the criteria used in the classification of its stages, the predominant view of DR was that diabetes primarily affected the retinal microvasculature and this then causes secondary damage and degeneration. Although fundus examination and other imaging techniques are the primary and most useful method of diagnosis, they only show the damage inflicted on the retinal vasculature. Over the years, it has become apparent that this is only part of the damage caused by

diabetes in the retina. New models of experimental diabetes and cell biology techniques have shown that the neural retina is also affected early in diabetes (Antonetti et al., 2006) (Figure 4) and, through the use of electroretinogram (ERG) recording techniques, vision impairments are detected earlier than the vascular changes (Barber, 2003), suggesting that neural changes are a result of diabetes and not a consequence of BRB breakdown (Antonetti et al., 2006). Therefore, DR can more accurately be defined as a neurovascular disease.

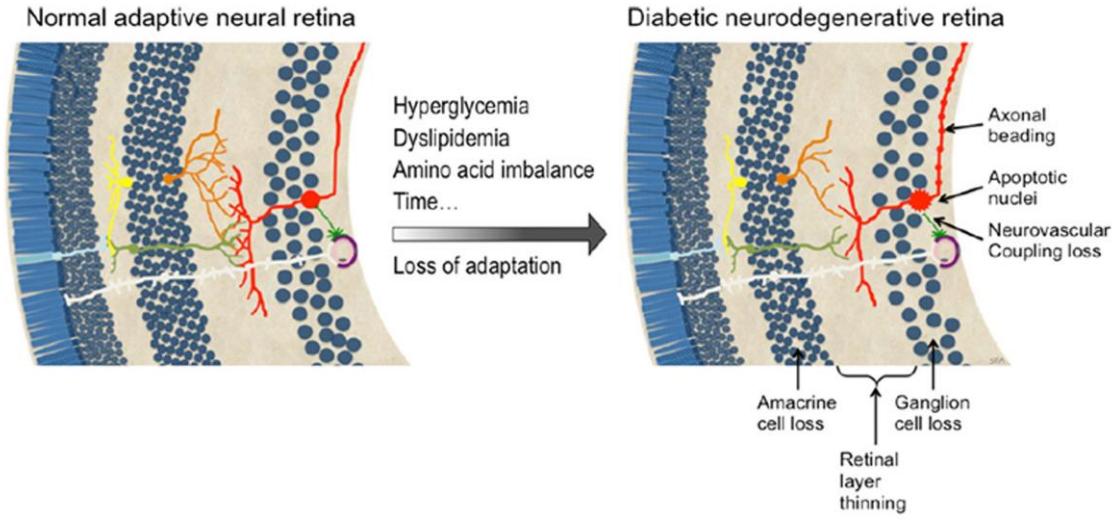

Figure 4: DR changes to the neural retina. With increased disruptions to the metabolic homeostasis, retinal neuronal cells begin to lose adaptation and the first signs of neurodegeneration begin to appear. According to color, the cell types are: light blue – photoreceptor; white – Müller cell; yellow – horizontal cell; light green – bipolar cell; orange – amacrine cell; red – RGC; dark green – astrocyte; light pink – endothelial cell; purple – pericyte. (Adapted from Abcouwer and Gardner, 2014, with permission)

In fact, DR shares many similarities with other neurodegenerative diseases, such as alterations in glutamatergic system (Santiago et al., 2006a, 2006b; Castilho et al., 2012), apoptosis (Park et al., 2003; Santiago et al., 2007; Leal et al., 2009), glial activation (Barber et al., 2000), and inflammation (Costa et al., 2009; C. A. Aveleira et al., 2010; Costa et al., 2012). Studies showed a correlation between the toxic levels of extracellular glutamate and the increased damage and higher apoptotic levels in retinal

neurons observed in diabetic conditions (Kowluru et al., 2001; Kusari et al., 2007). The altered levels of several neurotrophic factors in diabetic conditions can also exacerbate the damage occurring in retinal neurons, affect glucose metabolism and contribute to the inflammatory environment (Krabbe et al., 2007; Abu El-Asrar et al., 2013; Mysona et al., 2013).

1.2.1.3 Inflammation in DR

A chronic inflammatory environment is a characteristic of DR, especially in the earlier, pre-proliferative stages. Diabetic conditions can affect the physiologic repair mechanisms in tissues, and disrupt what is, in normal conditions, a pathway to protect cells against stress and increase cell survival. Indeed, in those conditions, there is an increased expression of several cytokines and growth factors that would improve neuronal cell function and survival in the short term such as VEGF, insulin-like growth factor-1 (IGF-1), interleukin (IL)-1α and tumor necrosis factor (TNF) (Gariano and Gardner, 2005). However, in diabetic conditions, this up-regulation is maintained for too long and becomes dysfunctional, contributing to increased vascular damage, neuronal degeneration, and to the development of retinopathy. Therefore, in early stages of DR there are several changes occurring that are usually associated with chronic inflammatory conditions such as an increase in leukostasis, microglial cell activation, increased action of nuclear factor-κB (NF-κB) leading to the amplified production and release of pro-inflammatory cytokines, chemokines and other inflammatory mediator proteins (Miyamoto et al., 1999; Barber, 2003; Krady et al., 2005). Cytokines such as the above mentioned TNF, IL-1β and IL-6 are increased in DR and connected to retinal leukostasis, BRB breakdown and the higher levels of apoptosis present in DR (Adamis and Berman, 2008; Costa et al., 2012). Several chemokines, such as MCP-1 and SDF-1 (Chen et al., 2010; Panee, 2012; Nawaz et al., 2013), and other proteins, such as nitric oxide (NO) synthases (Zheng and Kern, 2009), are also implicated in several

inflammatory processes of DR. The inhibition of this 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).

1.2.1.4 Origins of DR

Hyperglycemia has traditionally been considered the major cause in the initiation and progression of DR but, over the years, several studies revealed that high glucose levels alone may not be enough to cause the variety of cellular and physiological alterations occurring in DR (Figure 5) (ADVANCE Collaborative Group et al., 2008; Ismail-Beigi et al., 2010).

Hyperglycemic conditions dysregulate several pathways in the retina: overuse of glycolytic pathways, hexosamine pathways and an increased production of reactive oxygen species (ROS). There are also high levels of advanced glycation end-products (AGEs), due to an increased use of nonenzymatic glycosylation. These alterations have been shown to lead to neovascularization, cell death by apoptosis, oxidative stress and inflammation, contributing to retinal damage in diabetes (Ola et al., 2012).

Beyond hyperglycemia, several studies show that hyperlipidemia is also involved in the development of DR. A high content of plasma lipids in diabetic patients can increase the risk of DR (Ansquer et al., 2009), although the mechanisms behind this are not fully understood. Several hypotheses have been proposed, mainly related to the alterations in the levels of several elements such as acylcarnitine, ceramides, ketone bodies, oxidized fatty acids, polyunsaturated fatty acids and sphingolipids (Ola et al., 2012). Furthermore, a high-fat diet can also increase the level of oxidative stress and the intensity of the inflammatory response in the retina (Antonetti et al., 2006; Kowluru and Chan, 2007).

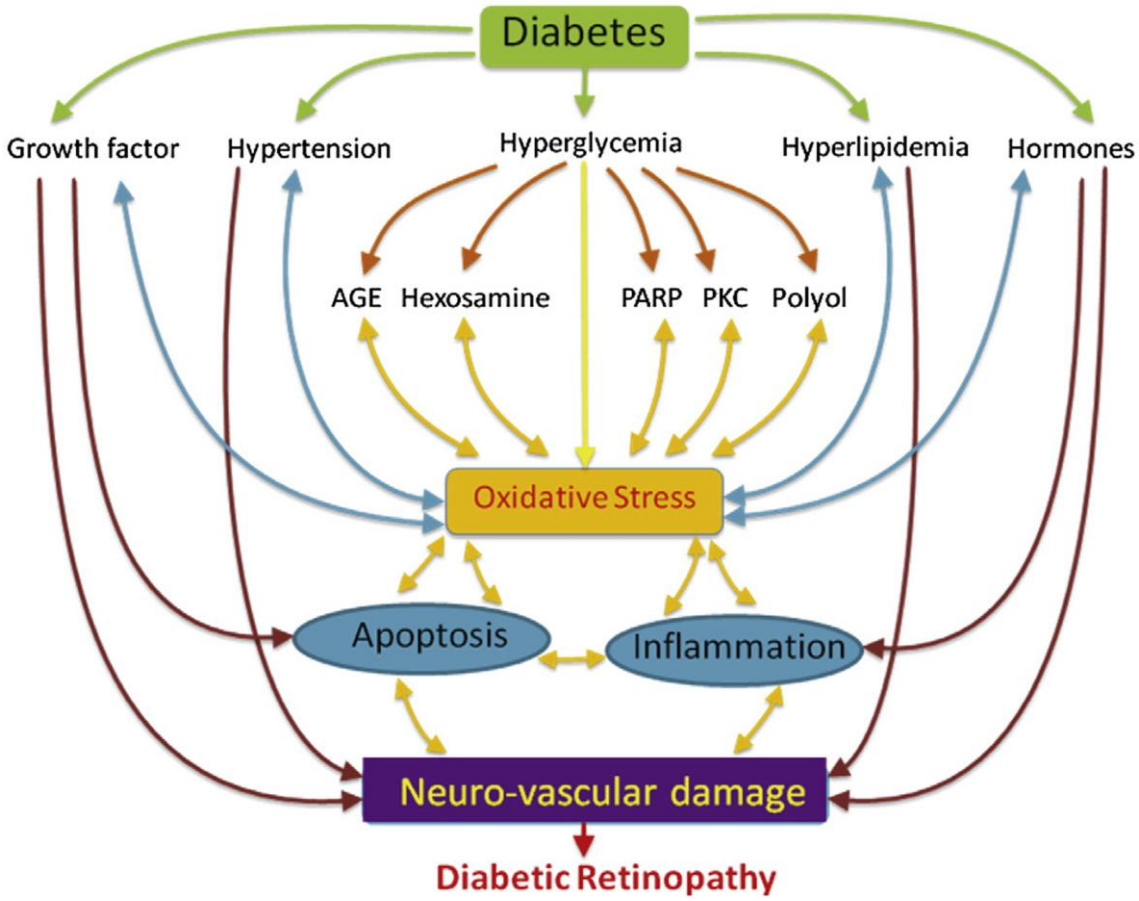

Figure 5: Schematics of the complex origins of DR. Diabetic conditions can induce the activation of numerous pathways that lead to neuronal and vascular damage in the retina. (From Ola et al., 2012, with permission)

Dysregulation of the renin angiotensin system (RAS) is also implicated in the development of DR. Several components of RAS are increased in patients with proliferative DR, including Angiotensin II, a peptide that can act as a growth factor, promoting apoptosis and exacerbating the harmful effects of AGEs, adding to the pathogenesis of DR (Nagai et al., 2007; Satofuka et al., 2009). Hormone dysregulation also plays a role in DR, with alterations in the levels of several hormones besides insulin being detected in diabetic patients. Excessive levels of IGF-1 are present in DR, and lead to serious vascular and glial complications through an increase in VEGF (Wilkinson-Berka et al., 2006); Growth hormone is also connected to retinal neovascularization and its inhibition may have protective effects in DR (Harvey et al.,

2009; Wilkinson-Berka et al., 2006); Aldosterone can be released in the retina in response to angiontensin II, among other stimuli, and is capable of inducing angiogenesis, inflammation and oxidative stress, conditions that may implicate aldosterone in the pathology of DR (Wilkinson-Berka et al., 2009); The levels of adrenomedullin, a vasodilator peptide, were elevated in diabetic patients and can also contribute to the pathogenesis of DR (Caliumi et al., 2007).

1.3 The Adenosinergic System

1.3.1 Adenosine

Adenosine is a purine nucleoside essential to all living cells. Beyond its role in energy metabolism and genetic transmission of information, adenosine is also a messenger that regulates numerous physiological processes in several tissues, particularly in the cardiovascular and nervous systems. 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 (Dunwiddie and Masino, 2001), although its actions can be more complex and dynamic, particularly in the CNS and in inflammatory conditions. 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 inflammatory responses and in neuroprotection against ischemia and epilepsy (Dunwiddie and Masino, 2001; Wardas, 2002; Sebastião and Ribeiro, 2009). Beyond physiological roles, adenosine also has a connection to several recreational drugs such as alcohol, opiates, and most notably, caffeine (Dunwiddie and Masino, 2001; Sebastião and Ribeiro, 2009). Several components are involved in this signaling system, from receptors that convey the signal transmitted by adenosine, to several enzymes and nucleoside transporters that regulate both the

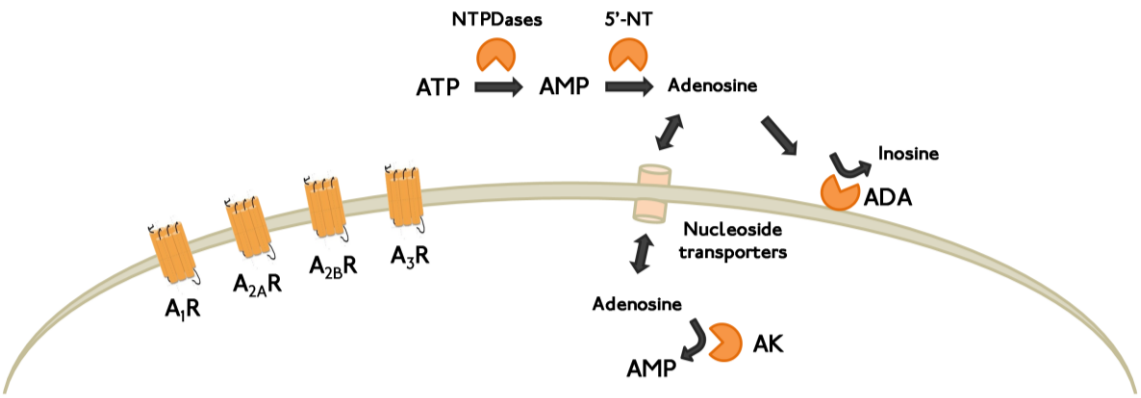

Figure 6: General schematics of the adenosinergic system. Adenosine can be released into the extracellular space by the nucleoside transporters, or by the purine degradation chain, that converts ATP, ADP and AMP to adenosine. The adenosine receptors are activated and convey the signal to intracellular pathways and regulate membrane channels. Adenosine is then removed from the extracellular space through degradation by ADA or through uptake by the nucleoside transporters where, once inside the cell, adenosine can be removed by AK.

extracellular and intracellular adenosine levels, maintaining a basal level of signaling in normal conditions (Figure 6).

1.3.2 Adenosine receptors

The actions of adenosine are mediated by selective receptors located in the cell membrane. Based on differences in molecular structure and pharmacological profile, four different receptors have been cloned and characterized (Ralevic and Burnstock, 1998): A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) (Figure 7). All these receptors belong to the superfamily of G protein coupled receptors (GPCR), presenting seven transmembranar 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 the brunt

of signaling is mediated by coupling to G-proteins, some G-protein-independent effects have also been reported (Ralevic and Burnstock, 1998; Haskó et al., 2008).

Historically, adenosine receptors have been usually classified according to their affinity for adenosine into high affinity (A_1R and $A_{2A}R$) and low affinity ($A_{2B}R$ and A_3R). However, the determination of receptor affinity has always been difficult, due to how fast adenosine is formed and metabolized in biological preparations, leading to different and sometimes contrasting results (Gallo-Rodriguez et al., 1994; Dunwiddie and Masino, 2001), according to the methods used. Therefore, the most reliable data could be considered the assessment of potency of adenosine in functional assays, and they reveal that the A_3R, although still low affinity in rodents, is a high affinity receptor in humans (Fredholm et al., 2011). To further complicate the situation, the potency of endogenous adenosine effects may not only be dependent on receptor affinity, but also on the number of receptors available (Arslan et al., 1999) and the type of response measured, due to the amplification steps the signaling pathways undergo after receptor activation.

The adenosine receptors are abundant throughout the organism, being present in most tissues and cell types, particularly in the CNS where these receptors can be found in all brain areas (Sebastião and Ribeiro, 2009). In the retina, most neuronal cells express A_1R and/or $A_{2A}R$, with retinal ganglion cells also expressing A_3R (Housley et al., 2009; Li et al., 2013; Huang et al., 2014); Müller cells have all but one receptor type present, with no direct A_3R detection so far (Wurm et al., 2011), while astrocytes and microglial cells express all four receptors (Gebicke-Haerter et al., 1996; Hammarberg et al., 2003; Haskó et al., 2005; Koscsó et al., 2012); pericytes express A_1R and $A_{2A}R$ (Grant et al., 1999; Lutty and McLeod, 2003), endothelial cells express $A_{2A}R$ and $A_{2B}R$ (Kvanta et al., 1997; Housley et al., 2009), while epithelial cells can express all four receptors (Kvanta et al., 1997; Housley et al., 2009; Wan et al., 2011).

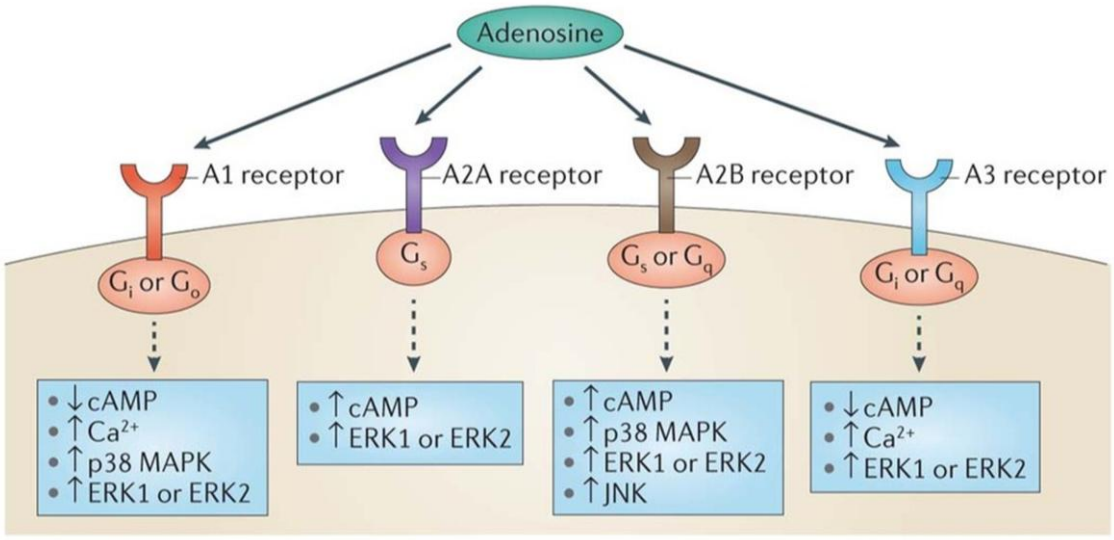

Figure 7: The adenosine receptors. Extracellular adenosine can activate four different GPCRs: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. $A_{2A}R$ and $A_{2B}R$ can stimulate adenylyl cyclase, while A_1R and A_3R can inhibit it. A_1R and A_3R can induce the release of Ca^{2+}, while all four receptors can interact with the MAPK family. (adapted from Antonioli et al., 2013, with permission)

1.3.2.1 A_1 adenosine receptor

A_1R has a very wide distribution, and is the most abundant adenosine receptor in the CNS (Dunwiddie and Masino, 2001). The receptor couples with G_i and G_o proteins and the most common signaling pathway is the inhibition of adenylate cyclase, leading to a decrease in the levels of the secondary messenger cAMP and consequently to a reduction in the activity of cAMP-dependent protein kinase A (PKA) (Dunwiddie and Masino, 2001). Beyond the direct effect on adenylate cyclase, this inhibition also counters the effect of other agents that stimulate adenylate cyclase (Wardas, 2002). A_1R activation can also activate members of the mitogen-activated protein kinase (MAPK) family, such as extracellular signal-regulated kinase (ERK) 1/2 and p38 (Schulte and Fredholm, 2003; Antonioli et al., 2013). Another signaling pathway triggered by A_1R is the activation of phospholipase C (PLC). Activation of this enzyme leads to production of diacylglycerol and inositol 1,4,5-triphosphate (IP_3), and the release of stored Ca^{2+} by interaction of IP_3 with specific receptors. The increase in

the intracellular Ca^{2+} levels may, in turn, activate other proteins and pathways, such as protein kinase C (PKC), NO synthase and Ca^{2+}-dependent K^+ channels (Dunwiddie and Masino, 2001). In some tissues, A_1R can circumvent this pathway, by interacting with other types of K^+ channels through G proteins (Dunwiddie and Masino, 2001). A_1R can also disrupt Ca^{2+} currents, by inhibiting certain membrane Ca^{2+} channels (Ralevic and Burnstock, 1998; Santos et al., 2000).

Overall, A_1R has a largely inhibitory action, leading to a state of reduced activity, particularly in neurons where it induces a decrease in neuronal excitability, firing rate and neurotransmitter release, contributing to the neuroprotective role of adenosine during hypoxia and ischemic conditions (Wardas, 2002; Sebastião and Ribeiro, 2009).

1.3.2.2 A_{2A} adenosine receptor

Like A_1R, $A_{2A}R$ is present has a wide presence, but with a more focused distribution, including immune tissues and the central and peripheral nervous system. This receptor couples with G_s protein mainly, though other G proteins may be involved, such as G_{olf} (Ralevic and Burnstock, 1998; Schulte and Fredholm, 2003). As such, the signal pathway most commonly triggered by $A_{2A}R$ 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 $A_{2A}R$ 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). On the other hand, several cAMP-independent signal pathways have also been proposed for $A_{2A}R$, including modulation of P-type and N-type Ca^{2+} channels, activation of a serine/threonine protein phosphatase and the activation of

MAPK family members such as ERK 1/2 (Ralevic and Burnstock, 1998; Schulte and Fredholm, 2003; Kreckler et al., 2006).

The role of $A_{2A}R$ in the CNS contrasts to role to A_1R. $A_{2A}R$ has a more excitatory function in neurons, being responsible for the potentiation of synaptic transmitter release (Sebastião and Ribeiro, 2009; Wardas, 2002). In other systems, however, it can also have inhibitory actions, such as playing an active role on the strong anti-inflammatory effects that adenosine has on most immune cells (Haskó et al., 2008).

1.3.2.3 A_{2B} adenosine receptor

$A_{2B}R$ has also a widespread distribution, but with a lesser density and, being a low affinity receptor (has a K_i in the μM range, as opposed to the other receptors, which have K_i in the nM range), requires higher concentrations of adenosine to elicit a response. These receptors couple with G_s and G_q proteins and activate adenylate cyclase, being also able to activate PLC and promote an IP_3-dependent increase of intracellular Ca^{2+} (Ralevic and Burnstock, 1998; Dunwiddie and Masino, 2001). $A_{2B}R$ is also able to interact with MAPK family members, such as ERK 1/2, JNK, and p38 (Schulte and Fredholm, 2003; Antonioli et al., 2013). The delay in the discovery of selective $A_{2B}R$ agonists and antagonists made the study of its physiological role challenging for a while. In blood vessels, $A_{2B}R$ are coupled to vasodilatation in both smooth muscle and endothelium and in the central nervous system they have been linked to increased neurotransmission (Feoktistov and Biaggioni, 1997). $A_{2B}R$ role in the immune system is somewhat contradictory: earlier studies revealed that $A_{2B}R$ activation increased the release of several pro-inflammatory messengers, such as IL-8 and IL-6, in different cell types, such as human mast cells (Feoktistov and Biaggioni, 1995) and astroglioma cells (Fiebich et al., 1996); later it was shown that $A_{2B}R$ activation in macrophages suppressed TNF expression and could have, with selective

agonists and in certain pathological situations, an effective anti-inflammatory role (Kreckler et al., 2006).

1.3.2.4 A_3 adenosine receptor

The A_3R 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). A_3R couples with G_i and G_q proteins and triggers PLC, promoting an 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 from their G-proteins, suggesting a role in the modulation of receptor activity (Macek et al., 1998). A_3R activation also mediates phosphorylation of protein kinase B and is able to decrease the levels of PKA (Fishman et al., 2002). A_3R can mediate phosphorylation of ERK 1/2, of the MAPK family, a signaling pathway involved in the modulation of microglia (Hammarberg et al., 2003; Schulte and Fredholm, 2003), and can also play a protective role in retinal ganglion cells, along with A_1R (Zhang et al., 2006).

1.3.2.5 Interactions 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 proximity of different adenosine receptors means adenosine can activate multiple signaling pathways and create a self-modulating environment, with $A_{2A}R$ and $A_{2B}R$ activating adenylate cyclase, and A_1R and A_3R inhibiting it. On the case of $A_{2A}R$ and $A_{2B}R$, the interactions may be synergistic, with the lower affinity $A_{2B}R$ supporting the

higher affinity $A_{2A}R$, 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: $A_{2A}R$ can decrease A_1R activity, either directly or by activation of adenosine transport which, in conditions of high neuronal activity and/or ATP release, means a decrease in available extracellular adenosine and therefore a decrease in A_1R activation (Sebastião and Ribeiro, 2009). A_3R is able to uncouple A_1R, via a PKC-dependent mechanism (Dunwiddie and Masino, 2001), and a study performed in avian retinal cells showed a connection between high levels of $A_{2A}R$ activation and an upregulation of A_1R, via a cAMP/PKA dependent pathway (Pereira et al., 2010).

1.3.2.6 Receptor dimerization

While monomeric receptors can activate signaling cascades (Chabre and le Maire, 2005), the adenosine receptors can form multimeric structures, from homodimerization to hetero-oligomer formation, usually in association with other GPCR's. There has been evidence to support the existence of A_1-A_1R and A_{2A}-$A_{2A}R$ homodimers in physiological conditions (Canals et al., 2004; Suzuki et al., 2009), of the heteromer A_1-$A_{2A}R$, which may influence the release of glutamate by adenosine (Ciruela et al., 2006), and of hetero-oligomers with other purinergic receptors (Yoshioka et al., 2002; Suzuki et al., 2006), dopaminergic receptors (Ginés et al., 2000; Canals et al., 2003; Vidi et al., 2008), cannabinoid receptors (Carriba et al., 2007) and glutamate receptor homomers (Cabello et al., 2009). Beyond association with GPCRs, the adenosine receptors (and their oligomers) can also have heteromerization with other proteins, namely adenosine deaminase (Torvinen et al., 2002) and calmodulin (Navarro et al., 2009).

1.3.3 Management of adenosine levels

In many tissues and organs, the basal concentrations of extracellular adenosine (which are around 30-300 nM in the brain (Rudolphi and Schubert, 1997)) are able to activate a considerable number of high-affinity A_1R, $A_{2A}R$ and also A_3R. Due to a lower density of A_3R, it has been suggested that the interactions between A_1R and $A_{2A}R$ set a basal purinergic balance that shows an equilibrium between the release and uptake of extracellular adenosine, a signal for homeostasis in the system (Dunwiddie and Masino, 2001; Fredholm, 2007).

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. Similarly, there are also two main methods for the removal of extracellular adenosine: 1) uptake across the cell membrane by neurons and neighboring glial cells and; 2) the deamination to inosine by the extracellular form of the enzyme adenosine deaminase (ADA).

1.3.3.1 Extracellular conversion of adenine nucleotides

There are several mechanisms for adenine nucleotide release, and they vary according to the regions of the CNS and methods of stimulation used (Latini and Pedata, 2001). ATP is co-localized with neurotransmitters such as acetylcholine, dopamine, serotonin and norepinephrine, and is co-released upon electrical stimulation (Zimmermann, 1994). Extracellular ATP then activates P2 receptors and is quickly converted to adenosine, serving a dual purpose: removal of ATP and activation of other downstream receptors (Latini and Pedata, 2001). cAMP can also be released into the extracellular space, through a non-specific energy-dependent transporter, providing another source for adenosine production (Henderson and Strauss, 1991). cAMP can also be converted to 5'-AMP inside the cells, which is then released and becomes a

source of adenosine, a possibility raised upon observation of a NMDA-evoked adenosine release in rat cortical slices (Craig et al., 1994). Furthermore, a study recently identified AMP as a full agonist of A_1R, suggesting that there may be an alternate form of activation of A_1R compared to the other receptors, when there is an ATP-dependent adenosine release (Rittiner et al., 2012).

Several extracellular enzymes handle the conversion of released adenine nucleotides. From nucleoside triphosphate diphosphohydrolases (NTPDase) to ecto-nucleotide pyrophosphatase/phosphodiesterases and apyrases, virtually all adenine nucleotides can be dephosphorylated to 5'-adenosine monophosphate (5'-AMP) fairly quickly, which is then dephosphorylated to adenosine by 5'-ectonucleotidase (5'-NT) (Dunwiddie and Masino, 2001). All of them are present in the CNS (Dunwiddie and Masino, 2001; Latini and Pedata, 2001), including the retina (Iandiev et al., 2007; Ricatti et al., 2009), where Müller cells, in particular, have this enzyme chain best characterized (Iandiev et al., 2007; Wurm et al., 2008).

These enzymes have a rather broad specificity, are generally rapid in their action (Dunwiddie and Masino, 2001) and 5'-NT, being the converging step in this degradation chain, has a particularly relevant role. It is a homodimer linked to the plasma membrane through a glycosyl-phosphatidylinositol lipid anchor, and is inhibited by adenosine triphosphate (ATP) and adenosine diphosphate (ADP). Because of this, 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 cytotoxic effects, and increasing protective actions of adenosine (Braun et al., 1998; Latini and Pedata, 2001).

1.3.3.2 Adenosine transport and release

The other mechanism that can increase extracellular adenosine levels is the release of intracellular adenosine by bi-directional nucleoside transporters, although there is some evidence to support the existence of a mechanism for the direct release of adenosine in the cerebellum of mice, through adenosine-filled vesicles (Klyuch et al., 2012).

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); and concentrative nucleoside transporters that use the Na^+ gradient to provide energy for the transport (Parkinson et al., 2011). The equilibrative transporters have been classified according to their sensitivity to the selective inhibitor S6-(4-nitrobenzyl)-mercaptopurine riboside (NBMPR), and four isoforms have been characterized, ENT1 to 4. They mediate a facilitated diffusion of nucleosides in both directions across the plasma membrane, transporting naturally occurring nucleosides with broad selectivity. All of them are widely distributed, but ENT2 levels are higher in skeletal muscle and brain, while ENT4 has a more marked presence in heart, liver and brain (Choi and Berdis, 2012). The concentrative transporters, consisting of three characterized isoforms (CNT1-3), perform a secondary active transport function, where the inward transport of a nucleoside is coupled with an inwardly directed transmembrane sodium gradient. Of the three, only CNT2 is expressed significantly in brain tissues (Choi and Berdis, 2012). As for the retina, studies have shown the expression of ENT1-2 and CNT1-2 in whole retina extracts from rats (Nagase et al., 2006) and also a rat Müller cell line (Akanuma et al., 2013). In a rat retinal capillary endothelial cell line, the expression of ENT1-2 and CNT2-3 was also detected (Nagase et al., 2006).

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 (Dunwiddie and Masino, 2001). Intracellularly, adenosine can be either phosphorylated to AMP by AK or deaminated to inosine by ADA. Both enzymes are present in rat retina (Vindeirinho et al., 2013). Due to its lower Km (2 μM for AK while ADA features a Km of 17-45 μM, in rat whole brain), AK becomes easily saturated with basal concentrations of adenosine, which are in the nanomolar range. Because of this, it is probable that phosphorylation by AK is the main pathway of adenosine metabolism in normal physiological conditions, while deamination by ADA may be more relevant in pathological conditions, when adenosine concentrations rise (Dunwiddie and Masino, 2001; Latini and Pedata, 2001). In those conditions, the flux shifts and the equilibrative nucleoside 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).

1.3.3.3 Removal of extracellular adenosine

To ensure the proper function of the adenosine signaling pathways, extracellular adenosine must be cleared or inactivated afterwards to prevent unwanted receptor activation. The primary mechanism of adenosine removal is its uptake across the cell membrane of neurons and neighboring glial cells, by use of the nucleoside transporters previously described. Although most of the extracellular adenosine is cleared by reuptake in normal conditions, deamination of adenosine to inosine by extracellular ADA also constitute another mechanism (Dunwiddie and Masino, 2001). It is worth noting that inosine, by itself, can act as an incomplete agonist for the A_3R (Fredholm et al., 2001; Jin et al., 1997). ADA 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_1R (Torvinen et al., 2002) and $A_{2B}R$ (Antonioli et al., 2014). During hypoxia and ischemia 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).

1.3.3.4 Regulation of intracellular adenosine

The intracellular levels of adenosine can affect the extracellular concentrations, due to the action of the equilibrative transporters, making the regulation of the enzymes involved in the adenosine cycle critical.

There are two soluble 5'-NT found in the cytosol: one is inosine monophosphate (IMP)-selective, while the other has an affinity 15-20 times higher for AMP than IMP. Since the Km 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, which occurs during high metabolic activity, is required to activate these enzymes (Latini and Pedata, 2001). Since the intracellular concentration of ATP exceeds 50 times that of AMP, 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.

Beyond the action of 5'-NT, another source of adenosine comes from the hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase, in a pathway that is 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 (Latini et al.,

1995). Since this reaction is reversible, SAH hydrolase can also function as a possible pathway for adenosine removal, 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).

1.4 The adenosinergic system in diabetes

Various forms of stress and pathological conditions, from ischemia and hypoxia to epilepsy, can affect the adenosinergic system (Fredholm, 2007). Diabetes, being a prolonged condition that causes metabolic alterations to the cellular environment, was likely to influence the adenosinergic system as well. Indeed, several studies have investigated the effect of diabetes and hyperglycemia on adenosinergic system components in various tissues and models.

The adenosine receptors, the key transmitters of adenosine signaling, have altered levels in several tissues under diabetic conditions, such as brain, liver and heart (Grdeń et al., 2005; Pawelczyk et al., 2005; Duarte et al., 2006; Grden et al., 2007). In the same manner, several of the enzymes involved in the regulation of adenosine levels are also very sensitive to metabolic and homeostatic alterations. Both nucleotide degrading (NTPDases, 5'-NT) and adenosine degrading (ADA, AK) enzymes were shown to be affected by diabetes in several tissues (Pawelczyk et al., 2000; Lunkes et al., 2004; Kocbuch et al., 2009; Schmatz et al., 2009; Lee et al., 2011).

Despite these studies, only in recent years did the focus shift to the retina, to reveal the extent of the effect that diabetic conditions may have on the adenosinergic system in the retina, and previous work in our group has contributed to this body of knowledge.

1.4.1 Adenosine and Diabetic Retinopathy

Changes to the adenosinergic system in the retina have been reported in retinal neural cells exposed to elevated glucose concentrations (HG) and in diabetic animals. Our previous studies have supplied most of the information pertaining to the protein levels of adenosine receptors and ADA (Vindeirinho et al., 2013).

We have revealed that the expression and density of A_1R suffer alterations in diabetic conditions in the retina: Protein levels of this receptor are increased in rat retinal cell cultures subjected to HG, and the density of A_1R is also increased in rat retinas after four weeks of diabetes (Vindeirinho et al., 2013). However, when diabetes was sustained for a longer period of time (12 weeks) another group observed a decrease in the expression of this receptor (Lau et al., 2013).

$A_{2A}R$ has become a focus of study in the diabetic retina, mainly due to its potential role in the inflammatory conditions in early DR. In mouse, rat and porcine, this receptor is shown to be up-regulated in diabetic conditions, both in retinal cell cultures and diabetic retinas (Elsherbiny et al., 2013a, 2013b; Vindeirinho et al., 2013).

As for the A_3R, our study showed that the density of this receptor shows a transient increase in diabetic rat retinas, after one week of diabetes, and a significant decrease after four weeks of diabetes. The $A_{2B}R$, on the other hand, showed no alterations to the protein levels in rat retinal cells under HG and in diabetic rat retinas (Vindeirinho et al., 2013).

Several studies have revealed alterations to the enzymes of the adenosinergic system in the retina in diabetic conditions: One study performed in the retina of diabetic rats showed that adenosine is heavily involved in the control of osmotic glial swelling during diabetes, and that diabetic conditions prompt a differential expression of NTPDase1, which hydrolyzes both ATP and ADP in equal measure, from being restricted to blood vessels in control animals to being present in retinal glial cells in diabetic animals (Wurm et al., 2008). The same study revealed an increase in mRNA levels of

NTPDase1 in diabetic retinas, while NTPDase2 and 5'-NT expression were the same. In diabetic rat Müller cells, glutamate induces the extracellular generation of adenosine, through 5'-NT (Wurm et al., 2008), probably due to the alterations in expression and distribution of NTPDase1, which drives an increase in AMP availability for degradation by 5'-NT.

Our study showed that, in diabetic rat retinas after four weeks, ADA protein levels are decreased (Vindeirinho et al., 2013). The same study revealed a significant decrease in ADA activity in retinal cultures under HG, despite no alterations to the protein levels of the enzyme. Another study showed that, in the diabetic retinas of mice, AK protein levels were decreased after eight weeks of diabetes, and this decrease is reduced by the inhibition of AK itself (Elsherbiny et al., 2013a).

In a study performed in T2DM patients with DR, an increase in plasma levels of adenosine was observed, but not in patients without DR, leading the authors to suggest adenosine as a biomarker for the diagnosis of DR (Xia et al., 2014). Another factor that may affect extracellular adenosine levels is the increase in ATP release. In fact, it was reported in rat retinal neural cells cultured with elevated glucose concentration that the depolarization-evoked release of ATP increases. The same study demonstrated that ATP degradation is impaired in the same conditions (Costa et al., 2009).

These changes occurring at several levels of the adenosinergic system in the diabetic retina may result in functional alterations to the processes controlled or modulated by adenosine.

The osmotic swelling of glial cells present in diabetic retinas is prevented by administration of adenosine (10 μM) in rats, but this prevention is suppressed by using 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), an A_1R antagonist (Wurm et al., 2008), while the blockade of $A_{2A}R$ had no effect. This suggests that the action of adenosine is mediated by activation of A_1R, which leads to an opening of potassium and chloride channels in the outer membrane of glial cells. The same study showed that the endogenous purinergic signaling that prevents swelling under osmotic stress is inactive

in diabetic conditions, although the receptors involved are functional and the mechanism works upon adenosine administration (as mentioned above).

The potential anti-inflammatory action of adenosine was evaluated in the retina: in retinal microglia cultures, exposure to Amadori-glycated albumin (AGA) triggers the inflammatory response seen in diabetic conditions, namely the increased expression and release of TNF, and this effect of AGA was successfully blocked by activation of $A_{2A}R$ in both mice (Ibrahim et al., 2011), rat (Elsherbiny et al., 2013a) and porcine (Elsherbiny et al., 2013b) retinal microglial cells. In mice, the activation of $A_{2A}R$ with 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzenepropanoic acid (CGS21680), a selective agonist, also reduced TNF levels and decreased cell death in diabetic retinas (Ibrahim et al., 2011).

These results point out to a protective, anti-inflammatory effect of adenosine, and $A_{2A}R$ activation in particular. This protective effect however, is contentious, as is the role of $A_{2A}R$ activation in general, with at least one other study showing conflicting results: The treatment of rMC-1, a rat Müller cell line, incubated in hyperglycemic-like conditions with either ATP or adenosine scavengers reduced the pro-inflammatory caspase-1 activation (Trueblood et al., 2011). A non-selective adenosine receptor antagonist (DPCPX at a concentration of 10 μM) also reduced caspase-1 activation, which was mimicked with an $A_{2B}R$ selective antagonist. Taken together, the existing data on the role of adenosine in the retina during diabetes remains controversial and the effects of the modulation of this system most likely will depend on the target receptors and/or cell types, and be influenced by the experimental approach.

1.5 Objectives

DR is the most common complication of diabetes, and the leading cause of preventable blindness in working-age adults worldwide. DR is commonly considered a disease of the retinal microvasculature, but this concept is being increasingly

challenged: the current knowledge of the early stages of DR, with particular highlights to neurodegenerative and inflammatory features intrinsic to the disease, strongly supports to the notion that DR is much more than a microvascular disease. Adenosine, with its role as a metabolic stress sensor and as modulator of neurotransmission and inflammation, may be a crucial player in the excitotoxic and inflammatory environment characteristic of the early stages of DR. There is evidence of alterations in several components of the adenosinergic system in several organs and tissues under diabetes, including adenosine receptors and enzymatic activity. Recently, some progress has been made, mainly by our group, to elucidate the effects of diabetes on the adenosinergic system in the context of DR. However, it is of utmost importance to further elucidate the impact of diabetic conditions on the retinal adenosinergic system. This may open new avenues for the identification of new potential therapeutic targets on the adenosinergic system.

For this reason, in Chapter 3 we aimed to study the impact of diabetes or prolonged exposure to high glucose on different components of the retinal adenosinergic system, namely on the adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R), the enzymes responsible for the extracellular generation of adenosine, the enzymes and nucleoside transporters responsible for reducing adenosine levels, and adenosine levels.

The dipeptidyl peptidase 4 (DPP4) inhibitors began being used in 2006 ("2006 - FDA Approves New Treatment for Diabetes," n.d.) as effective oral hypoglycemics, nowadays having widespread use in treatment of diabetes. DPP4 can have a strong interaction with ADA, whose activity levels are strongly decreased in HG conditions. In Chapter 4 we sought to better understand the influence of DPP4, and the effects of DPP4 inhibition, on ADA activity in HG conditions.

Adenosine receptor modulation has been studied for several neurodegenerative conditions with promising results in several pathologies, including Parkinson's Disease and Huntington's Disease, mainly through A_1R and $A_{2A}R$ receptor modulation. In DR, some studies have addressed this potential for modulation, focusing on $A_{2A}R$, with

somewhat conflicting results. Therefore, in Chapter 5, we sought to uncover a potential protective role for the modulation of adenosine signaling in DR.

In summary, DR is a multifactorial disease and adenosine signaling works not only as a sensor of homeostasis, but also modulates numerous processes that are affected by DR. Therefore, in this work we intended on uncover the effects of DR in the adenosinergic system and to understand how this system can be used as a potential protective target in the early stages of 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 Chemical Co. (St. Louis, MO, USA). Trypsin (UPS grade) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) was obtained from GIBCO/Life Technologies Inc. (Grand Island, NY, USA). Hoechst 33342 was obtained from Invitrogen-Molecular Probes (Leiden, The Netherlands). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Madrid, Spain). Sodium dodecyl sulfate (SDS) solution 10%, bromophenol blue, bis-acrylamide solution 30% were acquired from Bio-Rad Laboratories (München, Germany). Low-fat dry milk used was from Nestlé (Vevey, Switzerland). Enhanced chemifluorescense substrate (ECF) was purchased from GE Healthcare (Hertfordshire, UK). Primers used for qRT-PCR were synthesized by MWG Biotech (Ebersberg, Germany). The iScript cDNA synthesis kit and the iQ SYBR Green supermix were purchased from Bio-Rad Laboratories (München, Germany). BCA Protein Assay Kit was acquired from Thermo Scientific (Waltham, MA, USA). Protein A agarose resin and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dako fluorescent mounting medium was from Dako (Glostrup, Denmark) and optimal cutting temperature compound (OCT) was from Sakura® Finetek (Leiden, The Netherlands). Anestocil (oxybuprocaine), Tropicil Top (tropicamide) and Opticol (sodium hyaluronate) were purchased from Laboratório Edol – Produtos Farmacêuticos SA (Linda-a-Velha, Portugal).

All other reagents were obtained from Fisher Scientific, Sigma-Aldrich and Merck & Co. (White House Station, NJ, USA). All solutions were aqueous if not described, expect for phenylmethanesulphonylfluoride (PMSF) which was prepared in dimethyl sulfoxide (DMSO).

Table 1: Antibodies used in Western Blot and Immunohistochemistry studies

	Host	Type	Dilution Western Blot	Dilution Immuno histochemistry	Origin
Primary antibodies					
Anti-ADA	Rabbit	Polyclonal	1:500	-	Santa Cruz Biotechnology
Anti-AK	Goat	Polyclonal	1:500	-	Santa Cruz Biotechnology
Anti-DPP4	Rabbit	Polyclonal	1:1000	-	Abcam
Anti- Brn3a	Mouse	Polyclonal	-	1:500	Merck Millipore
Anti-β actin	Mouse	Polyclonal	1:20000	-	Invitrogen
Secondary antibodies					
AP-conjugated Anti-rabbit	Goat	Polyclonal	1:20000	-	GE Healthcare
AP-conjugated Anti-mouse	Goat	Polyclonal	1:20000	-	GE Healthcare
AP-conjugated Anti-goat	Donkey	Polyclonal	1:5000	-	Santa Cruz Biotechnology
Alexa Fluor 568 Anti-mouse	Goat	Polyclonal	-	1:250	Invitrogen

Table 2: List of substances used in retinal cell culture incubations

Name	Description	Final concentration	Origin
DPP4	Recombinant mouse DPP4/CD26	5 µg/ml	R&D Systems
Vildagliptin	DPP4 inhibitor	2 nM	Novartis Pharma
CGS 21680	$A_{2A}R$ selective agonist	30 nM	Tocris Bioscience
SCH 58261	$A_{2A}R$ selective antagonist	50 nM	Tocris Bioscience
2'-MeCCPA	A_1R selective agonist	50 nM	Tocris Bioscience
DPCPX	A_1R selective antagonist	50 nM	Tocris Bioscience
NBMPR	ENT1 selective inhibitor at low concentrations	0.1 µM	Tocris Bioscience

2.2 *In vitro* studies

2.2.1 Isolation and culture of rat retinal cells

Retinal cell cultures were obtained from new-born (3-5 days old) Wistar rats as previously described (Santiago et al., 2006a). Each culture was made from a single litter of 8 to 12 animals. Briefly, pups were decapitated, eyes removed, and retinas were dissected under sterile conditions in a Ca^{2+} and Mg^{2+}-free Hank´s balanced salt solution (CMF, in mM: 32 NaCl 5.4 KCl, 0.45 KH_2PO_4, 0.45 Na_2HPO_4, 4 $NaHCO_3$, 2.8 glucose, 5 HEPES, pH 7.1). The retinas were then subjected to digestion in CMF supplemented with 0.05% trypsin (w/v), at 37ºC for 12-15 minutes. After this incubation the tissue was gently centrifuged (130 g/min). The resulting supernatant was discarded and the pellet resuspended in MEM, buffered with 25 mM HEPES and 26 mM $NaHCO_3$, pH 7.4, and supplemented with 10% heat-inactivated FBS (v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml). The tissue was then mechanically dissociated using a 5 ml glass pipette. After dissociation, cellular viability and density were determined by a viable cell count using Trypan Blue staining. Cells were diluted 1:50 in CMF with 0.4% Trypan Blue and placed in a hemocytometer under an 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, or multi-well plates containing glass coverslips with 16 mm diameter, both coated with poly-D-lysine (0.1 mg/ml). Cells were then maintained in MEM at 37 °C, in a humidified atmosphere with 5% CO_2. After two days, the culture medium was supplemented with 25 mM D-glucose in addition to the 5 mM already present in MEM, reaching a final concentration of glucose 30 mM to simulate high glucose (HG) conditions in diabetes, or with D-mannitol 25 mM (plus 5 mM glucose), which was used as an osmotic control (Figure 8). The concentration of glucose in control conditions was 5 mM. The cells were used for experimentation after seven days of HG exposure. The incubations required for several experimental procedures are detailed in Table 2.

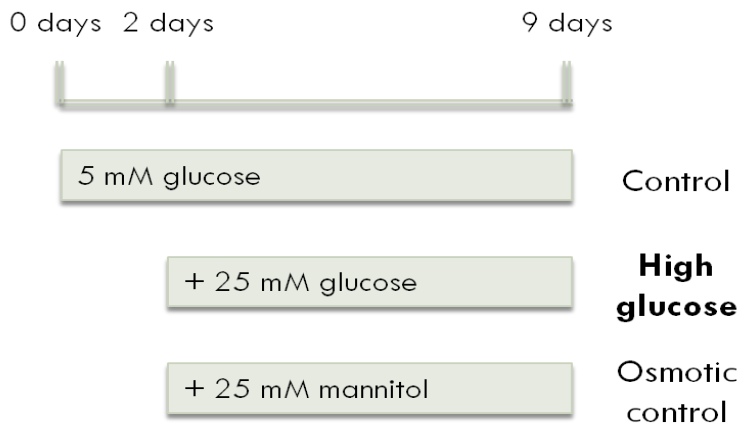

Figure 8: Representation of the retinal cell culture incubations for the control, HG and osmotic control groups.

2.2.2 SDS-PAGE and Western Blot

After nine days in culture, cells cultured in 6-well plates were removed from the incubator and rapidly placed on ice. The culture medium was removed and cells were washed three times with ice-cold Krebs buffer solution (in mM: 111 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2. KH_2PO_4, 2.5 $CaCl_2$, 24.8 $NaHCO_3$, 11.1 glucose, 15 HEPES, pH 7.4), and whole cell extracts were prepared by scrapping the plates in 80 µl of lysis buffer (137 mM NaCl, 20 mM Tris, 10% Nonidet P-40, 10% glycerol) supplemented with protease and phosphatase inhibitors (0.1 mM PMSF, 1 µg/ml CLAP 1, 1 mM DTT, 1mg/ml orthovanadate, 1 M NaF), 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. 1/5 of sample volume of 6x concentrated sample buffer was added (0.5 M Tris-HCl, 30% glycerol, 10% SDS, 600 mM DTT, 0.012% bromophenol blue) and the extracts were denatured with heat (95 °C for ten minutes).

Proteins were separated in a 7.5% sodium dodecyl sulphate-polyacrilamide gel (SDS-PAGE) using MiniPROTEAN® 3 systems (Bio-Rad Laboratories). 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 (25 mM Tris, 25 mM bicine, 0.1% SDS, pH 8.3) and electrophoresis was carried at 60 V for ten minutes and then 130 V until proper molecular weight separation was reached (30-40 min); the proteins were then electrotransfered (750 mA, for 150 min) to PVDF membranes, submerged in electrophoresis buffer (10 mM CAPS, 10% methanol, pH 11 adjusted with NaOH), using Trans-Blot Cell apparatus (Bio-Rad Laboratories). After this procedure, membranes were immersed in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6 adjusted with HCl) containing 0.1% (v/v) Tween 20 (TBS-T) and 5% (w/v) low-fat dry milk for one 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, under continuous shaking (For antibody dilution and supplier, refer to Table 1). 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 one hour at room temperature. Membranes were again washed, and then dried before being resolved using ECF. The scanning was performed with the VersaDoc Imaging System (Bio Rad Laboratories), and the data analyzed with Quantity One (Bio Rad Laboratories).

For the loading control, membranes were stripped using 0.1 M glycine, pH 2.3 and then reprobed for β-actin for one hour at room temperature. The described protocol was then followed for the remainder of the technique.

2.2.3Total RNA extraction

After nine days in culture, total RNA was extracted from cells cultured in 6-well plates, using a column-based RNA purification kit (RNeasy Mini Kit, Qiagen) and following the manufacturer provided protocol for adherent cells. Isolated RNA was then eluted in RNase-free water, and RNA concentration and sample purity assessed by

UV-visible spectroscopy in an automated NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The NanoDrop also provided the ration of absorbance at 260 and 280 nm (A260/280), as a measure of purity of isolated nucleic acid and indicator of protein contamination, and the ratio of absorbance at 260 and 230 nm (A260/230), as a indicator of contamination with phenolates and thiocyanates resulting from the isolation protocol. Samples were considered acceptable for cDNA synthesis when ratio values for both A260/A280 and A260/A230 were between 1.8 and 2.2, respectively. Total RNA integrity was assessed using the Experion system (Bio-Rad Laboratories) and evaluating the peak of the 18S and 28S ribosomal RNA bands. In order to avoid genomic DNA contamination, samples were treated with DNase I (1 U of DNase/ 1 µg of RNA) for 30 minutes at 37ºC, before cDNA synthesis. DNase was inhibited by adding EDTA to a final concentration of 2.5 mM and inactivated by heating samples at 65ºC for 15 minutes.

2.2.4 cDNA synthesis and qRT-PCR

cDNA was synthesized using the iScript-cDNA synthesis (Bio-Rad Laboratories), following the manufacturer's instruction. 1 µg of RNA was used as template. The resulting cDNA, diluted from 1:1 to 1:10, was used for the amplification of the desired genes using the SYBR Green PCR Master Mix (Bio-Rad Laboratories), which contains all the necessary components for real-time quantitative polymerase chain reaction (qRT-PCR). The reactions were carried out in triplicate, following a 3-step protocol consisting of a 10 s denaturation step at 95 °C, followed by 30 s at the annealing temperature optimal for each primer, and lastly a 30 s step at 72ºC for elongation. This protocol was performed with the IQ5 Multi-Color Real-Time PCR Detection System (Bio-Rad Laboratories). The PCR reaction-mix contained 2 µl of cDNA, specific primer set (2.5 µM each), 4µl of RNase-free water and 10 µl of SYBR Green PCR Master Mix in a final volume of 20 µl. A standard curve for all sets of

primers was obtained by a series of 10-fold dilutions, and the primer-pair efficiency calculated. All primers used had an amplification efficiency of 90-105%. The PCR products for each gene were analyzed by melting-curve and a standard agarose gel electrophoresis. Data from the target genes was normalized using the expression of three stable reference genes (RNA polymerase 2, RNApol2; Peptidylprolyl isomerase A, Ppia; hypoxanthine guanine phosphoribosyl transferase 1, Hprt1) and the mRNA level ratios calculated using the altered Pfaffl model described previously (Hellemans et al., 2007), for normalizations with multiple reference genes. The primer sequences and amplicon size for each gene can be found on Table 3.

Table 3: List of primers used for qRT-PCR

Gene	Primer Sequence	Gene	Primer Sequence
A_1AR	(f) GATACCTCCGAGTCAAGATCC	**ENT1**	(f) GCCAACTACACAGCCCCCACTA
	(r) AAACATGGGTGTCAGGCC		(r) TCAGCAGTCACAGCAGGGAACAA
A_{2A}AR	(f) TCTTCGCCTGTTTTGTCCTG	**ENT2**	(f) CCTACAGCACCCTCTTCCTCAGT
	(r) ACCTGTCACCAAGCCATTG		(r) CCCAGCCAATCCATGACGTTGAA
A_{2B}AR	(f) GCTCCATCTTTAGCCTCTTGG	**CNT1**	(f) CAACACACAGAGGCAAAGAGAGTC
	(r) TCTTGCTCGTGTTCCAGTG		(r) CCACACCAGCAGCAAGGGCTAG
A_3AR	(f) TCTTCACCCATGCTTCCATC	**CNT2**	(f) GGAAGAGTGACTTGTGCAAGCTTG
	(r) CAGAAAGGACACTAGCCAGC		(r) GTGCTGGTATAGAGGTCACAGCA
ADA	(f) GAATCCCAAAACGACGCATG	**CNT3**	(f) CTGTCTTTTGGGGAATTGGACTGC
	(r) CACGGTGGACTTGAAGATGAG		(r) CCAGTAGTGGAGACTCTGTTTGC
AK	(f) AGAAAGACGCAGGCTCTTC	**Brn3a**	(f) ACCCTGTTCGTTTCAAATAG
	(r) AAGACAGGGAAAGCAGTGAC		(r) AGCGTTTATCCCTGGTAAT
TNF	(f) CCCAATCTGTGTCCTTCT	**IL-1β**	(f) ATAGAAGTCAAGACCAAAGTG
	(r) TTCTGAGCATCGTAGTTGT		(r) GACCATTGCTGTTTCCTAG
RNApol2	(f) CTTCAATCGTCAGCCAACTTTG	**Ppia**	(f) TTTGGGAAGGTGAAAGAAGGC
	(r) GCATTGTATGGAGTTGTCACAC		(r) ACAGAAGGAATGGTTTGATGGG
Hprt1	(f) CCTTGACTATAATGAGCACTT	Sequence in 5' – 3' order;	
	(r) GCCACATCAACAGGACTC	(f) – forward (r) - reverse	

2.2.5 Enzymatic activity assays

Cell lysates were prepared in 50 mM Tris-HCl, supplemented with 0.2 mM PMSF and 1 µg/ml CLAP, 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.

2.2.5.1 NTPDase assay

The NTPDase assays were carried out in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µl as described by Battastini *et al.* (Battastini et al., 1991). Briefly, 20 µl of sample was added to the reaction medium and pre-incubated at 37°C, for 10 min, after which the reaction was initiated by the addition of ATP or ADP, at a final concentration of 1 mM, and allowed to proceed for 20 min in both cases. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA), to obtain a final concentration of 5%, and the assay tubes were chilled on ice for 10 min. Blank controls were carried out by adding the cell lysates after TCA addition, to correct the results for non-enzymatic nucleotide hydrolysis and normalize for variations between samples. The released inorganic phosphate (Pi) was assayed by the method of Rockstein and Herron (Rockstein and Herron, 1951), with ferrous sulfate-molybdate as colorimetric reagent and KH_2PO_4 as a standard. Briefly, 400 µl of ultra-pure water and 800 µl of sulfate-molybdate were added to the 400 µl of the reaction mix, and allowed to rest for five to ten minutes, after which samples were quantified spectrophotometrically at a wavelength of 660 nm. All samples were quantified in triplicate.

2.2.5.2 5'-NT assay

The activity of 5'-NT was determined using the method of Heymann *et al.* (Heymann et al., 1984), were cell lysates were placed in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris-HCl buffer, pH 7.5, in a final volume of 200 µl. 20 µl of sample was added to the mixture and pre-incubated at 37 °C, for 10 min, after which the reaction was initiated by the addition of AMP, at a final concentration of 2 mM, and allowed to proceed for 20 min. The following steps of the protocol, as well as quantification of released Pi, were the same ones described previously for NTPDase activity.

2.2.5.3 ADA assay

The activity of ADA was determined according to Guisti and Galanti, (Giusti 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. 100 µl of the samples were added to 500 µl of a solution of 21 mM of adenosine in 50 mM phosphate buffer [4.73 g/L $NaH_2PO_4(H_2O)$, 5.62 g/L $Na_2HPO_4(H_2O)_{12}$], pH 6.5 and were incubated at 37 °C for 60 min. For a standard, a solution of ammonium sulphate [75 mM $(NH_4)_2SO_4$ in phosphate buffer] was used, and for a reagent control, phosphate buffer was used. Afterwards, 1.5 ml of a phenol solution (106 mM phenol, 170 µM sodium nitroprusside) and a sodium hypochlorite solution (11 mM NaOCl, 125 mM NaOH) were added and the samples incubated at 37 °C for 30 min. Blank controls were carried out by adding the cell lysates before this last incubation and after phenol solution addition, to correct the results for non-ADA related ammonia content and normalize for variations between samples. The final products were quantified in triplicate spectrophotometrically at a wavelength of 620 nm. Results were calculated according to the formula: [(sample - sample control)/ (standard - reagent control)] x50.

2.2.6 Adenosine quantification

Cells were lysated with 0.6 M perchloric acid, supplemented with 25 mM EDTA-Na^+, and centrifuged at 14 000g for 2 min at 4 °C, according to previously described methods (Duarte et al., 2006). The resulting pellet was solubilized with 1 M NaOH for total protein analysis by the BCA method. After neutralization of the supernatant with 3 M KOH in 1.5 M Tris, the samples were centrifuged at 14000g for 2 min (4 °C). To determine extracellular accumulation of adenosine, medium was recovered prior to the above procedure. The resulting supernatants and medium samples were assayed for adenosine concentration by separation in a reverse-phase high-performance liquid chromatography (HPLC), with detection at 254 nm. The HPLC apparatus was a Beckman-System Gold with a computer controlled 126 Binary Pump Model and 166 Variable UV detector. The column used was a Lichrospher 100 RP-18 (5 µm) from Merck. An isocratic elution with phosphate buffer 10 mM (NaH_2PO_4; pH 6.0) and 14% methanol was performed with a flow rate of 1.5 ml/min, and each analysis took five minutes. Adenosine was identified by retention time, absorption spectra and correlation with standards.

2.2.7 Co-immunoprecipitation

The co-immunoprecipitation assay used was a free antibody approach, using ADA as “bait” protein, and DPP4 as the “prey”. Cells were cultured in 60 mm petri dishes for nine days, after which they were placed on ice. The culture medium was removed, cells were washed three times with ice-cold Krebs buffer solution, and whole cell extracts were prepared by scrapping the plates in 100 µl of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% DOC). The suspension was then cleared of its insoluble fraction by centrifugation at 14000 g, 10 min, 4 ºC, and the

protein concentration was determined by the BCA method using the BCA protein kit. Part of the cell lysates was stored to serve as input at a further step, while 500 µg of total protein was used for each sample, and the total volume adjusted to 500 µl with RIPA. Samples were incubated with 30 µl of Protein A agarose resin (Santa Cruz) for one hour, at 4 ºC, to pull down and clear direct bindings to the beads. After centrifugation (14000g, 5 min, 4 ºC), 2 µg of α-ADA antibody was added to the sample, while 2 µg of the corresponding IgG was added to the negative control, for an overnight incubation at 4 ºC, with continuous rotation. Afterwards, 1:10 of Protein A agarose resin was added to both sample and negative control and kept rotating for two hours, at 4 ºC. Samples were centrifuged (14000g, 5 min, 4 ºC) and the supernatant discarded. This was repeated between the following washes: 3x with 500 µl of RIPA, once with 500 µl of RIPA supplemented with 500 mM NaCl, and a final wash with 500 µl of RIPA. After the final centrifugation, 2x concentrated sample buffer was added in equal volume to the Protein A resin added previously, and samples were denaturated at 95 ºC, for 10 min. The final step before normal SDS-PAGE proceeded was one last centrifugation (15700g, 5 min, 4 ºC), and samples were loaded in the gel with care, along with the input stored previously.

2.2.8 Propidium iodide assay

Cells cultured in multi-well plates containing glass coverslips with 16 mm diameter for nine days were carefully washed twice with warm (37 ºC) phosphate buffer saline (PBS) consisting of: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4. Cells were then incubated with 500 µl of a solution of 2 µg/ml propidium iodide (PI) and 1 µg/ml Hoechst 33342 in PBS, at 37ºC for 10 min. After incubation cells were again carefully washed four times with warm PBS, to remove PI and Hoechst surplus, mounted on microscope glass slides over a drop of Dako fluorescent mounting medium, and immediately visualized with the fluorescence

microscope Zeiss Axioshop 2 Plus, coupled to the digital camera Axiocam HRc. 12 fields were acquired per coverslip, and counted using Fiji software.

2.3 *In vivo* studies

2.3.1 Diabetes induction in Wistar Rats

Experiments were performed according to the European Council directive 86/609/EEC and the legislation Portaria n. 1005/92, issued by the Portuguese Government for the protection of animals used for experimental and other scientific purposes. Animal handlers and the researchers are credited by the European Federation for Laboratory Animal Research (FELASA) category C or B for animal experimentation. In this work we have used a model of type 1 diabetes generated by STZ injection. STZ is synthesized by *Streptomycetes achromogenes*, and causes the destruction of pancreatic β-cells due to its selective uptake by the low affinity glucose transporter GLUT2 (Wang and Gleichmann, 1998) and the high cellular toxicity resulting from oxidative stress (Szkudelski, 2001).

Eight weeks old male Wistar rats, purchased from Charles River Laboratories (Spain), were rendered diabetic by a single intraperitoneal (i.p.) injection of STZ, (65 mg/kg body weight diluted in 10 mM sodium citrate, pH 4.5). 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. Diabetic status (blood glucose levels exceeding 250 mg/dL) was confirmed two days later with a glucometer (Ascencia Elite, Bayer, Portugal). The animals were maintained on a regular chow diet, *ad libitum,* for two different time periods: one week and four weeks, after which the animals were sacrificed under anesthesia [ketamine hydrochloride (50 mg/kg, Imalgene^{TM}, Merial, Porto Salvo, Portugal) and xylazine (10 mg/kg, Rompun^{TM}, Bayer, Leverkusen, Germany)] and/or procedures realized.

2.3.2 Treatment with SCH 58261

Four groups of aged-matched (eight weeks) Wistar rats were used for this set of experiments (Figure 9). After diabetes induction to the animals of two groups, they were maintained on a regular chow diet, *ad libitum,* for three weeks. At that time, one control group (non-diabetic) and one diabetic were subjected to daily i.p. injections of the $A_{2A}R$ selective antagonist SCH 58261 at the efficacious dose of 0.05 mg/kg (Canas et al., 2009), in saline (0.9% NaCl) with 10% DMSO, for seven days. The other two groups, one control and one diabetic, were subjected to daily i.p. injections of vehicle (0.9% NaCl with 10% DMSO), for seven days.

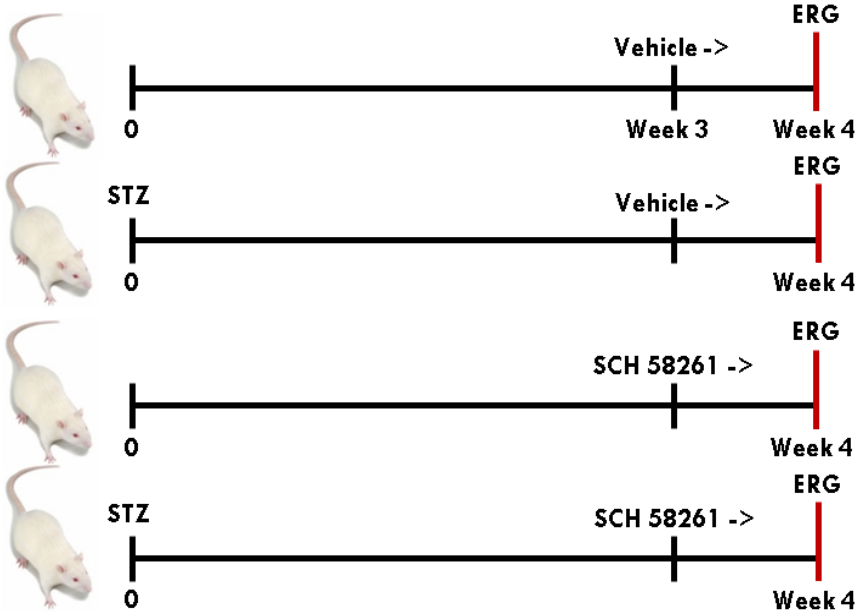

Figure 9: Representation of the experimental groups for the ERG procedures, and other experiments performed with rats treated with SCH 58261.

2.3.3 Whole retina extracts and Western Blot

The animals were euthanized by anesthesia (50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine) followed by decapitation. The eyes were removed and the retinas extracted and placed in 200 µl of the previously described lysis buffer, supplemented with protease inhibitors, 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), and the protocol described in 2.2.2 followed.

2.3.4 Total RNA extraction

Total RNA extraction was performed by an adaptation of the acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987), using TRI reagent (Sigma-Aldrich). Retinas were extracted and lysed in 1 ml TRI reagent, and homogenized by continuous pipetting, after which samples could be stored at -80ºC if necessary. RNA was isolated by phase separation by adding 0.2 ml chloroform to the samples, which were then shaken and allowed to rest for 15 minutes at room temperature. After centrifugation at 12 000 g for 15 minutes, 4 ºC, the upper aqueous phase containing RNA was collected and transferred to a new tube. RNA was precipitated by adding 0.5 ml of isopropanol and centrifuging samples at 12 000 g, ten minutes, 4 ºC. The supernatant was discarded and the RNA pellet was washed twice with 75% ethanol, by vortexing samples after adding ethanol and centrifuging at 7 500 g for five minutes, 4 ºC. After the final wash, ethanol was carefully removed, and the RNA pellet dried softly before adding 20-30 µl of RNase-Free water. Afterwards, all the procedures were similar to those described in 2.2.4.

2.3.5 Serum collection and adenosine quantification

The blood from the animals was collected from the decapitated neck, immediately after the sacrifice, and allowed to coagulate at room temperature for 15-30 min. The blood clotting was then removed by centrifugation at 2000 g for 10 min, 4 ºC, and the serum quickly transferred to eppendorfs and aliquoted for storage at -80ºC to prevent freeze-thaw. The adenosine quantification procedure followed the protocol described in 2.2.6.

2.3.6 Cryosections and immunohistochemistry

Immediately following sacrifice, the eyes were enucleated and placed in cold PBS. Eyes were placed in 4% paraformaldehyde (PFA) in PBS for 1 h, at 4 ºC, and then positioned under a microscope for the removal of the cornea and lens. The resulting eye-cup was immersed in cold 4% PFA for another hour. Three washes of 15 min each with cold PBS were performed, followed by a final 30 min wash also with PBS. After this final wash, the eye-cups were placed in 15% sucrose in PBS, at 4 ºC, for one hour, and then moved to a 30% sucrose solution in PBS, 4 ºC, for another hour. The eye-cups were then placed in appropriate molds and immersed in a solution comprised of equal parts Optimal cutting temperature compound (OCT) and 30% sucrose in PBS, and stored at -80 ºC. Retinal cryosections of 16 µm were obtained using a Shandon cryostat (Thermo Scientific) with a spacing of five slices between, four slices per microscope glass slide, and stored at -20 ºC. For the immunohistochemistry, slides were defrosted and allowed to dry for 45 min at room temperature, after which cryosections were fixed with acetone at -20ºC for 10 min. Cryosections were re-hydrated with PBS two times for 5 min, or until OCT disappeared. Cryosections were delineated with a PanPen, dried for 1 min, and then permeabilized in PBS with 0.25%

Triton X-100, 50 µl/section, for 30 min, after which they were blocked with 50 µl/section of 10% normal goat serum in PBS, with 1% bovine serum albumin (BSA), for 30 min, in a closed humidified plastic container. The serum was removed with absorbing paper and sections were incubated with α-Brn3a antibody (see Table 1), in PBS-1% BSA, 50 µl/section, in a closed humidified plastic container overnight at 4 ºC. Cryosections were then washed three times with PBS, for 10 min each. After removal of excess liquid, sections were incubated with the secondary antibody, in PBS-1% BSA, 50 µl/section, in a closed humidified plastic container covered with aluminum foil, for one hour, at room temperature. After three 10 min washes with PBS, sections were incubated with DAPI 1:1000 in PBS, 50 µl/section, for 15 min in the dark, at room temperature. Slices were washed three times, for 10 min each, with PBS, the excess liquid removed, and sections were mounted with Dako fluorescent mounting medium, and covered with a coverslip. Cryosections were allowed to dry before application of nail varnish to seal the coverslips. Sections were evaluated with the fluorescence microscope, with image acquisition of the entire length of each slice, and analyzed with Fiji software.

2.3.7 ERG procedures

Animals were dark-adapted for 12 h before the recordings, for a complete dark adaptation, and the animal preparation was carried under dim red light. The animals were anesthetized by i.p. injection of ketamine hydrochloride (50 mg/kg, Imalgene™, Merial, Porto Salvo, Portugal) and xylazine (10 mg/kg, Rompun™, Bayer, Leverkusen, Germany), and oxybuprocaine (4 mg/ml, Laboratórios Edol, Linda-a-Velha, Portugal) anesthetic was applied topically to the eyes. The pupils were dilated with topical tropicamide (1%, Laboratórios Edol, Linda-a-Velha, Portugal). The eyelids were held with isolated clips to keep the eyes open, and a sodium hyaluronate solution (0.3%) was applied throughout the procedure to maintain stable corneal potential (impedance

< 5 kohm) and for hydration. The body temperature of the animals was maintained with a heating pad set to 37ºC during the procedure.

The electrical responses were recorded with gold wire electrodes placed at the cornea, reference electrodes placed behind the ears, and a ground electrode in the tail. Electrode impedance did not exceed 5 kohm during the entire procedure. The waveforms were recorded with a band width of 1 to 300 Hz and sampled at 3.4 kHz (except for flicker test, where a 0.65 kHz sampling rate was used) by a digital acquisition system (Roland Consult GmbH, Brandenburg, Germany), and they were analyzed with the RETIport software (Roland Consult GmbH, Brandenburg, Germany).

A Ganzfeld stimulator (Roland Consult GmbH, Brandenburg, Germany) was used to deliver light stimuli. Following recommendations for a toxicological screening ERG procedure (Rosolen et al., 2005), four different ERG responses were recorded, as follows (Figure 10):

Scotopic Luminance Responses: Series of white light flashes of seven different light intensities (0.0095 to 9.49 cd-s/m^2) were delivered three times at 0.1 Hz to the dark adapted animals.

Photopic Adaptometry: With a white background light (25 cd/m^2), bright white flashes (9.49 cd-s/m^2) were delivered three times at 1.3 Hz at the beginning, and 2, 4, 8, and 16 min afterwards to assess the light adaptation process.

Photopic Luminance Responses: With a white background light (25 cd/m^2), white flashes of seven different light intensities (0.0095 to 9.49 cd-s/m^2) were delivered three times at 1.3 Hz.

Photopic Flicker: With a white background light (25 cd/m^2), white bright flashes (3.00 and 9.49 cd-s/m^2) were delivered ten times at 6.3 Hz.

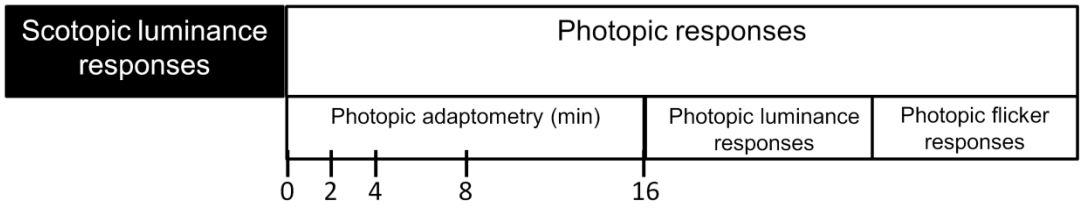

Figure 10: Representation of the light stimulation protocols. After at least 12 h of dark adaptation, animals were exposed to four sequential and different light stimulation protocols: Scotopic Luminance Responses, Photopic Adaptometry, Photopic Luminance Responses, and Photopic Flicker.

After recordings, several parameters were analyzed:

a-wave amplitude and time to peak: measured from the original waveform, from the graphic baseline to the negative a-wave trough. Time to peak was measured from the onset of light stimulus to the a-wave trough.

b-wave amplitude and time to peak: measured after a 50 Hz high frequency cut-off digital filter was used to isolate it from OPs, from the a-wave trough to the b-wave peak. Time to peak was measure from the onset of light stimulus to the b-wave peak.

Individual OP amplitude and time to peak: both amplitude and time to peak were measured as b-wave parameters. Low frequency cut-off digital filters (60 Hz in scotopic conditions, and 55 Hz in photopic conditions) were used to isolate OPs, according to OP main frequencies (Zhang et al., 2007), and the frequency spectrum of the original waveforms.

The use of digital filters to separate the b-wave from OPs is possible due to their difference in main frequencies (Zhang et al., 2007). OP components have higher main frequencies than the b-wave component, so a high frequency cut-off digital filter is able to isolate the b-wave, while a low frequency one is able to isolate the OPs. In the case of scotopic ERG, four OPs were identified and distinguished based on time to peak values (approximated values): OP1 (22-30ms), OP2 (34-46ms), OP3 (48-56ms), and OP4 (63-76ms). The time to peak values vary with flash light intensity, usually decreasing as the intensity of stimulus rises. Although minimal, three OPs were

identified and classified for the photopic ERG: OP2 (31-35ms), OP3 (42-50ms), and OP4 (57-65ms), based on similarities in time to peak values to scotopic ERG.

The b-wave was also fitted to the Naka-Rushton equation (Evans et al., 1993) which describes the dark-adapted ERG b-wave amplitude *R* of the form:

$$R/R_{max} = L^n/(L^n + K^n)$$

where *R* is the response to a luminance L, R_{max} is the asymptotic maximum response amplitude, *K* is the intensity that evokes a half-maximum response, and n represents a dimensionless variable that describes the slope of the intensity-response function.

Flicker responses were evaluated using the Fast Fourier Transform (FFT). For statistical analysis, the maximum amplitude and phase harmonic values were considered.

Chapter 3 Alterations of the key components of the adenosinergic system in the diabetic retina

3.1 Introduction

The signaling mechanisms activated by adenosine have a wide range of cellular responses, often producing opposite effects according to the situation, tissue, or cell type. This functional diversity is due to the effects of adenosine on the four different surface receptors: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R (Ralevic and Burnstock, 1998). The diverse portfolio of actions is maintained by strategic localization, differential adenosine affinity, and the modulation of several signaling cascades, although some signaling pathways are activated by more than one adenosine receptor or may converge downstream (Antonioli et al., 2013; Sheth et al., 2014). Because of this, adenosine signaling can often be contradictory, from inhibiting neurotransmitter release to inducing it, and going from neuroprotective to neurotoxic, and anti-inflammatory to pro-inflammatory (Ghiardi et al., 1999; Rogachev et al., 2006; Sheth et al., 2014). It is because of this often dual nature that, in pathological situations, it is necessary to observe the alterations that may occur to the levels of these receptors.

As stated above, adenosine receptor affinity levels also play a part in the differential effects of adenosine. Basal levels of extracellular adenosine are usually able to activate both A_1R and $A_{2A}R$, and also A_3R on occasion, while $A_{2B}R$ requires higher concentrations of the nucleoside (Dunwiddie and Masino, 2001; Fredholm, 2007). Adenosine levels can be increased or decreased in pathological situations, therefore affecting the features and intensity of the pathways activated. Because of this, it is necessary to determine not only the levels of adenosine, but also possible alterations to the density and activity of the key enzymes and transporters.

Therefore, to further understand the effects of DR in the adenosinergic system, we set out to study the expression of the adenosine receptors, the key enzymes and transporters, and also the enzymatic activity and adenosine levels in mixed rat retinal cell cultures and the retina of adult, diabetic rats.

In DR, Wurm *et al.* have shown that NTPDase1 presents an altered distribution in the rat retina, alongside an increased expression, while the expression and distribution of NTPDase2 and 5'-NT remained unaltered (Wurm et al., 2008). Our own previous work, also performed in rat retina and rat retinal cell cultures, showed the alterations that occur in the protein levels of the adenosine receptors, as well as the protein levels and activity of ADA (Vindeirinho et al., 2013). With these studies in mind, we focused our attention to the expression levels of the receptors, transporters, AK and ADA. We also assessed the enzymatic activity of the key enzymes beyond ADA, and measured the adenosine levels present in retinal cell cultures.

3.2 Results

3.2.1 Diabetes/high glucose alters the expression of adenosine receptors in retinal cell cultures and diabetic rat retinas

In order to determine if the expression levels of the adenosine receptors could account for the protein level profile we previously observed (Vindeirinho et al., 2013), we first exposed our retinal cell cultures to HG conditions (glucose 30 mM), to mimic hyperglycemic conditions, and evaluated the expression levels of the four adenosine receptors by quantitative RT-PCR. As illustrated in Figure 11, in cells subjected to HG, both A_1R and $A_{2A}R$ expression levels were increased after seven days of HG exposure: A_1R expression attained 1.22±0.04 fold of control ($p<0.01$), and $A_{2A}R$ increased over 1.81±0.17 fold of control ($p<0.001$). On the other hand, $A_{2B}R$ and A_3R expression levels were not altered in HG (1.11± 0.10 and 0.83±0.13 of control, respectively). The alterations occurring to A_1R and $A_{2A}R$ expression levels were not due to changes in osmolarity since incubation of cells with mannitol did not induced alterations in all adenosine receptors mRNA compared to control conditions.

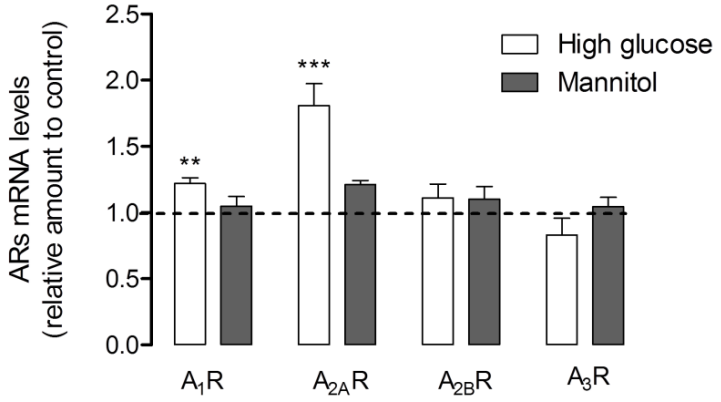

Figure 11: Adenosine receptor expression levels in cultured retinal cells. The expression levels of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R were evaluated by qRT-PCR, seven days after HG incubation. Experiments were carried out in triplicate. The mean±SEM of 4–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. **p<0.01, ***p<0.001.

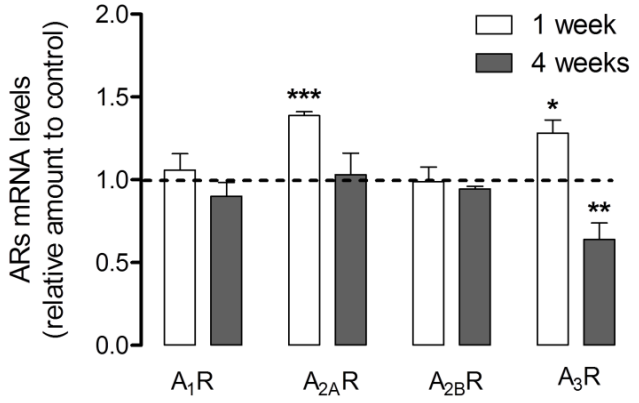

Figure 12: Adenosine receptor expression levels in diabetic retinas. The expression levels of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R were evaluated in the retina of diabetic rats by qRT-PCR, one and four weeks after diabetes induction. Experiments were carried out in triplicate. The mean±SEM of 4–5 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test.*p<0.05, **p<0.01, ***p<0.001.

To complement the experiments performed *in vitro*, the expression levels of the adenosine receptors were also evaluated in retinal extracts from diabetic rats and age-matched controls, at two different time points: one and four weeks after induction of diabetes. As seen in Figure 12, the mRNA levels of A_1R were not significantly altered at

one or four weeks (1.06±0.10 and 0.90±0.08 fold of control, respectively), while $A_{2A}R$ expression showed a significant increase at the first week, rising 1.39±0.02 in relation to control ($p<0.001$), before returning to control levels at four weeks (1.03±0.13 fold of control). $A_{2B}R$ expression levels remained unchanged at both time points of diabetes (0.99±0.09 fold of control at one week and 0.94±0.02 fold of control at four weeks). On the other hand, A_3R levels showed significant fluctuations: this receptor was increased 1.28±0.08 fold of control at one week of diabetes ($p<0.05$), but this increase was transient and, reaching the second time-point of four weeks, the expression levels of this receptor showed a significant decrease of 0.64±0.10 fold of control ($p<0.01$).

3.2.2 Diabetes/high glucose alters the expression of nucleoside transporters in retinal cell cultures and diabetic rat retinas

Previous studies have detected the presence of several nucleoside transporters in the retina: ENT1 and ENT2, as well as CNT1 and CNT2 were found in whole rat retina extracts, and CNT3 was further detected in a rat retinal capillary endothelial cell line (Nagase et al., 2006). Correspondingly, we analyzed the expression levels of these 5 transporters in retinal cell cultures under HG, and also in diabetic retinas at one and four weeks of diabetes.

As predicted by the previous studies and our own protocol for primary mixed retinal cell cultures, we could not detect the presence of CNT3 in our cultures which, due to the procedure of retinal isolation used, have a negligible presence of endothelial cells. However, and unlike the cited study, we could only detect trace amounts of CNT1 that were not high enough for proper quantification (Data not shown).

Again we exposed our retinal cell cultures to HG conditions, and evaluated the expression levels of the different NT's by quantitative RT-PCR. We observed a significant increase in the mRNA levels of ENT1, rising 1.37±0.1 above control levels, as seen in Figure 13, while the levels of ENT2 showed a tendency to increase as well,

although not significant (1.14±0.11 fold of control). CNT2 expression levels showed no alterations due to HG exposure (1.11±0.23 fold of control). These results were not influenced by osmotic effects, since cells incubated with mannitol retained levels similar to control cells.

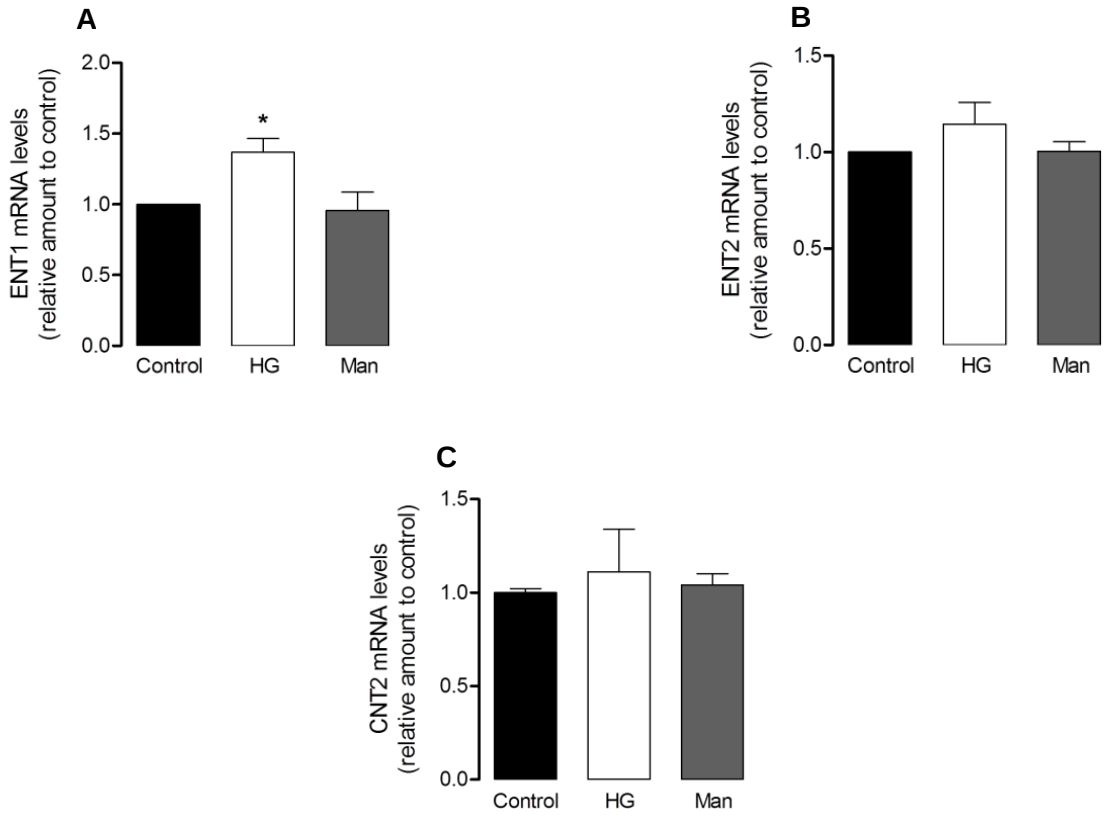

Figure 13: Nucleoside Transporter expression levels in cultured retinal cells. The expression levels of **(A)** ENT1, **(B)** ENT2, and **(C)** CNT2 were evaluated by qRT-PCR, seven days after HG incubation. Experiments were carried out in triplicate. The mean±SEM of 4–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. *p<0.05.

The expression levels of the nucleoside transporters were also evaluated in diabetic retinas where, as observed in retinal cell cultures, we could not detect CNT1 and CNT3 in enough concentration for proper quantification. As seen in Figure 14, ENT1 showed no alterations in expression neither at one, nor at four weeks of diabetes (1.06±0.19 and 0.94±0.13 fold of control, respectively). ENT2 also showed no significant alterations at both time points of diabetes, despite a tendency for a decrease at four

weeks (1.06±0.07 and 0.85±0.28 fold of control for one and four weeks, respectively). CNT2 showed a tendency for increased expression at one week (1.24±0.38 fold of control), but it only became significant at the fourth week time point, rising up to 1.86±0.39 fold of control ($p<0.05$).

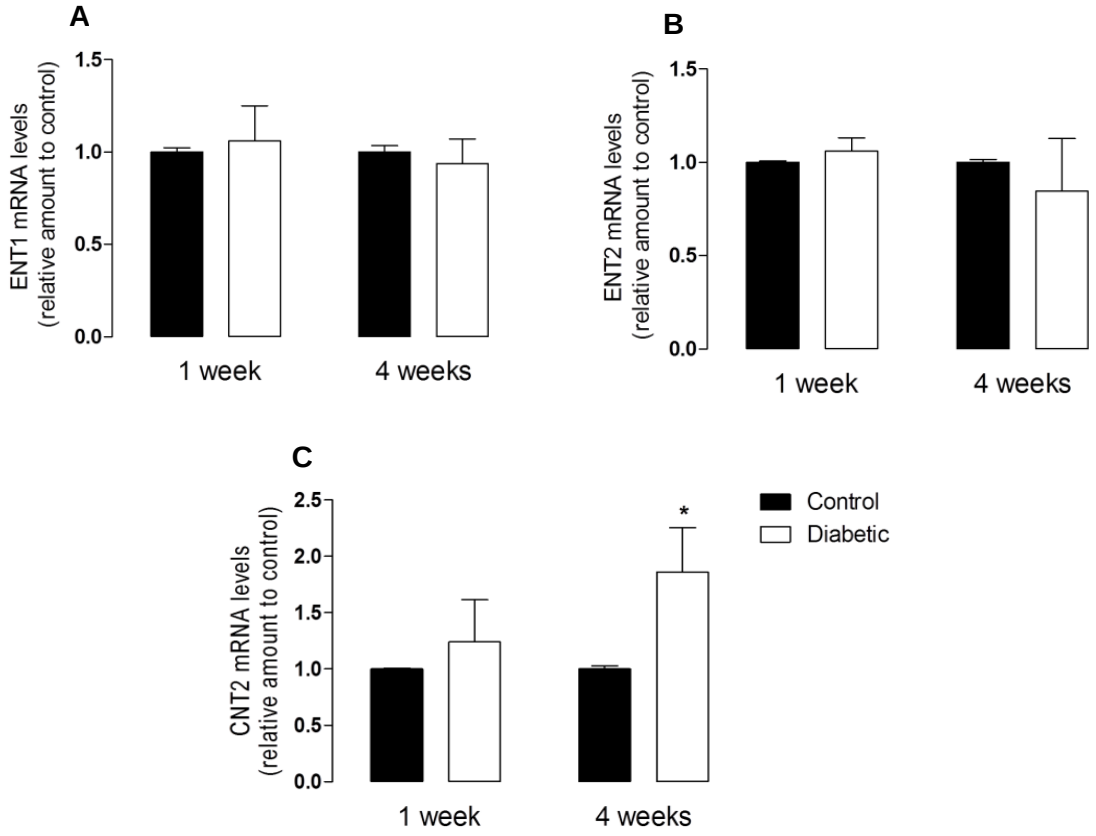

Figure 14: Nucleoside Transporter expression levels in diabetic retinas. The expression levels of **(A)** ENT1, **(B)** ENT2, and **(C)** CNT2 were evaluated by qRT-PCR, one and four weeks after diabetes induction. Experiments were carried out in triplicate. The mean±SEM of 4–5 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test.*$p<0.05$.

3.2.3 The expression levels of ADA are not altered in retinal cell cultures under HG, but are altered in diabetic rat retinas

As we did with the adenosine receptors, we sought to determine if the expression levels of ADA were in synchrony with the protein level profile revealed in

our previous studies (Vindeirinho et al., 2013). For that we assessed the mRNA levels of this enzyme in retinal cell cultures after seven days of HG exposure by qRT-PCR, and showed that, in accordance with the protein levels, ADA expression was unaltered in both HG conditions (1.02±0.20 fold of control, Figure 15A) and when exposed to the osmotic control.

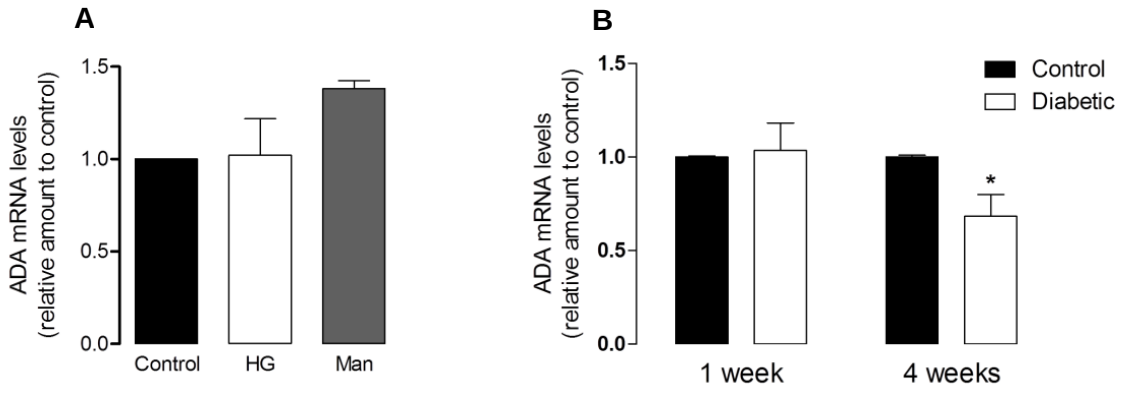

Figure 15: ADA expression levels in cultured retinal cells and diabetic retinas. (A) The expression levels of ADA were evaluated by qRT-PCR, in retinal cultures seven days after HG incubation. The mean±SEM of 4–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. **(B)** The expression levels of ADA were also evaluated in diabetic retinas one and four weeks after diabetes induction. The mean±SEM of 4–5 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test. Both experiments were carried out in triplicate. *p<0.05.

In diabetic rat retinas, the alterations occurring to the protein levels of ADA were also evident in the expression levels of this enzyme (Figure 15B), with no alterations showing at one week of diabetes (1.03±0.15 fold of control), but a significant decrease of 0.68±0.12 fold of control appearing by the fourth week time point (p<0.05).

3.2.4 The expression and protein levels of AK are not altered in retinal cell cultures under HG, but are altered in diabetic rat retinas

AK is the main enzyme responsible for the clearance of adenosine in the intracellular space, and its levels have been shown to suffer alterations in diabetic conditions (Pawelczyk et al., 2000). Therefore we set out to evaluate possible alterations to the protein levels of this enzyme in both retinal cell cultures under HG and diabetic retinas, by Western Blot, and alterations to the expression levels in the same conditions, by qRT-PCR.

As illustrated in Figure 16, in retinal cells after seven days of HG exposure, there were no alterations to the protein levels of AK (104.0±9.53% of control). When we analyzed the mRNA levels of this enzyme in the same conditions, we also found no alterations (1.04±0.04 fold of control), showing that, much like ADA, AK does not seem to be affected by HG conditions in retinal cells. In both experiments, the osmotic control had no effect on AK levels.

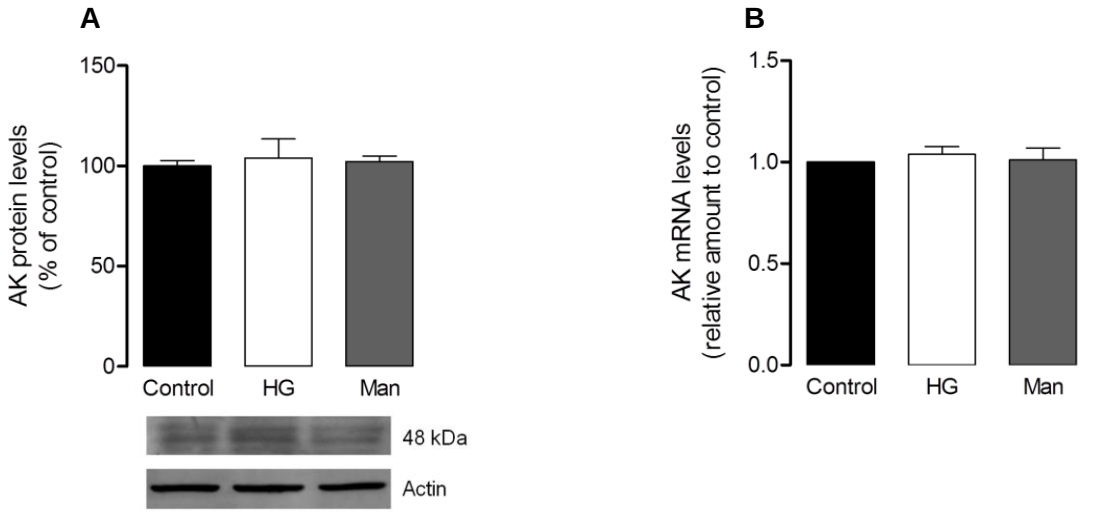

Figure 16: AK protein and expression levels in cultured retinal cells. (A) The protein levels of AK were evaluated by Western Blot analysis, seven days after HG incubation. The mean±SEM of 4–6 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. **(B)** The expression levels of AK were evaluated by qRT-PCR, seven days after HG incubation. Experiments were carried out in triplicate. The mean±SEM of 4–6 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test.

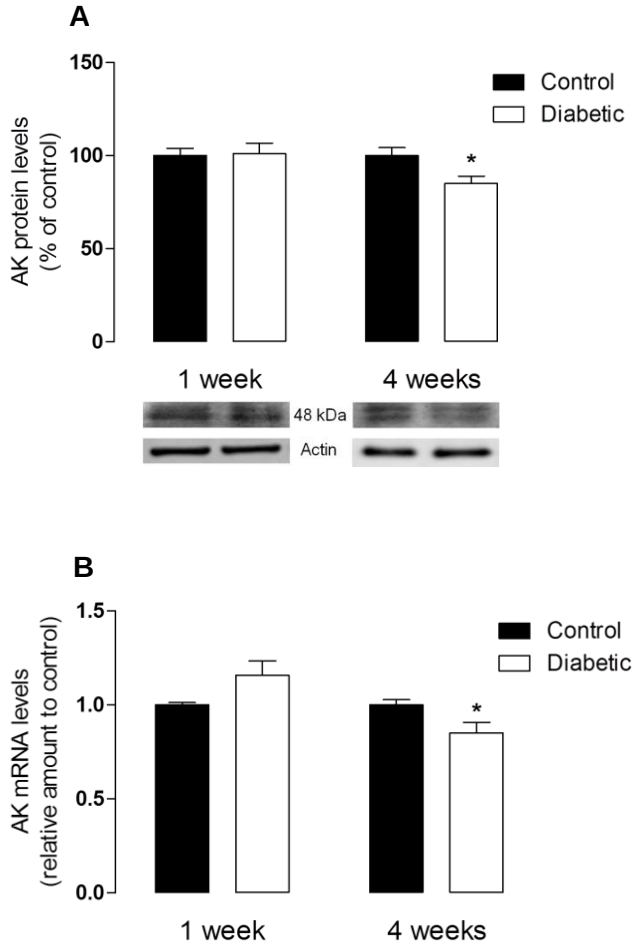

Figure 17: AK protein and expression levels in diabetic retinas. (A) The protein levels of AK were evaluated by Western Blot analysis, one and four weeks after diabetes induction. The mean±SEM of 4–7 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test.*$p<0.05$. **(B)** The expression levels of AK were evaluated by qRT-PCR, one and four weeks after diabetes induction. Experiments were carried out in triplicate. The mean±SEM of 4–5 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test.*$p<0.05$.

Both protein and expression levels of AK were also assessed in the retina of control or diabetic rats. As seen in Figure 17A, after one week of diabetes there were no alterations to the protein levels of AK in diabetic retinas (101.0±5.71% of control). These levels, however showed a significant reduction after four weeks of diabetes, decreasing to 85.0±3.95% of control ($p<0.05$). When we assessed expression levels in diabetic retinas (shown in Figure 17B), the results showed a similar pattern: no significant alterations after one week of diabetes, despite a small tendency for increase

(1.16±0.07 fold of control), and a significant decrease apparent after four weeks of diabetes, with expression reduced to 0.85±0.06 fold of control ($p<0.05$). This decrease is reminiscent of the alterations occurring to ADA levels also in diabetic retinas after four weeks of diabetes (Vindeirinho et al., 2013).

3.2.5 The enzymatic activity of NTPDases in retinal cell cultures is affected by HG conditions

It is known that diabetes conditions can affect the activity of purine degrading enzymes (Schmatz et al., 2009), and our own work has previously shown a substantial decrease in ADA activity in retinal cells under HG conditions (Vindeirinho et al., 2013). Furthermore, another study has shown that the distribution and expression levels of retinal NTPDase1 were altered in diabetic conditions (Wurm et al., 2008). Therefore, it becomes necessary to evaluate the levels of the activity of NTPDase1 and 2, and 5'-NT to have a clear image of the state of the purine degradation chain in HG conditions.
Using retinal cell cultures exposed to HG conditions for seven days, we performed an NTPDase activity assay on cell preparations where we measured the released Pi after incubation with either ATP or ADP. For this set of experiments, we removed mannitol from the procedure after a first confirmation that it was not affecting the results, for a more efficient use of sample material (data not shown). As shown in Figure 18A, there was a significant increase in the Pi levels following incubation with ATP in HG conditions, with values rising above 126.2±9.39% of control ($p<0.05$). ADP degradation showed an even higher increase in HG conditions of released Pi after ADP incubation, rising to 173.7±16.64% of control ($p<0.05$).

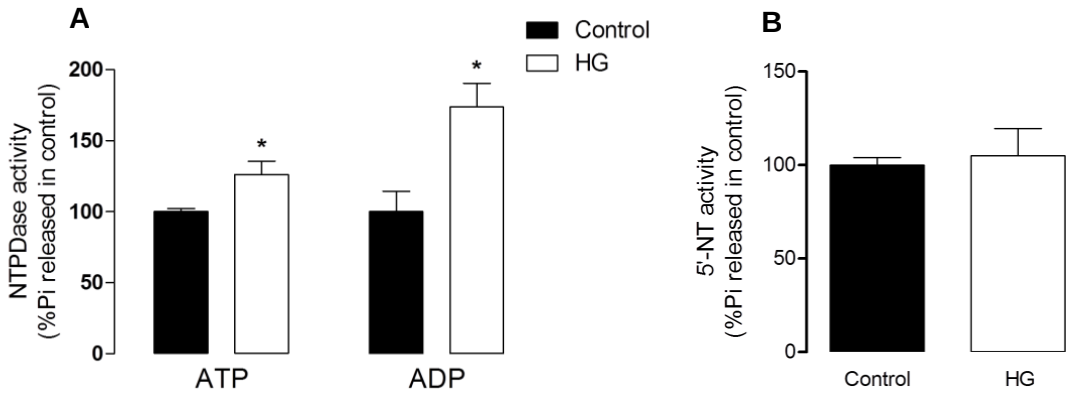

Figure 18: Effects of HG on the activity of NTPDases and 5'-NT (A) NTPDase activity was analyzed in retinal cultures after seven days of HG, by incubating the extracts with either ATP or ADP, to differentiate between NTPDase1 and NTPDase2. The activity was measured by the amount of P_i released, after normalization with the sample's protein content. The mean±SEM of 4–6 independent experiments was analyzed with the Student's t-test (HG vs. Control) and F test.*p<0.05. **(B)** 5'-NT activity was measured in retinal cells after seven days of HG, by incubating cells with AMP and analyzing the amount of P_i released, after protein normalization. The mean±SEM of 4–5 independent experiments was analyzed with the Student's t-test (HG vs. Control) and F test.

We also assessed 5'-NT activity in retinal cell cultures exposed to HG by incubating cell preparations with AMP and measuring the Pi released as a result. As seen on Figure 18B, the activity of 5'-NT was not affected by HG conditions, with Pi levels similar to control (105.1±14.31% of control). Despite this, the increase in ATP/ADP degradation would presumably increase the availability of AMP, 5'-NT's substrate of choice which, along with the previously described decrease in ADA activity, could have an impact on extracellular adenosine levels.

So we used retinal cell cultures exposed to HG conditions for seven days, and evaluated the levels of extra and intracellular adenosine, in collected medium and cytosolic fraction, by HPLC. We see in Figure 19 that HG conditions significantly increased extracellular adenosine levels, to 118.4±4.55% of control ($p<0.05$), and decreased intracellular levels to 55.12±9.32% of control ($p<0.001$). These alterations were not affected by the osmotic control, which retained levels similar to control.

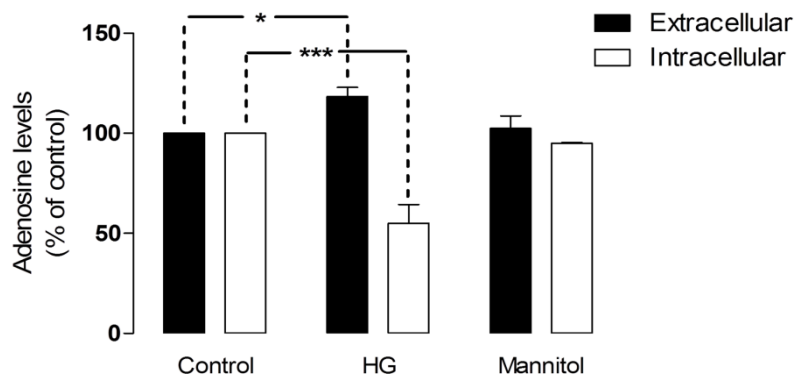

Figure 19: Adenosine levels in retinal cell cultures under HG. The levels of adenosine were measured in 20 µl of culture medium and intracellular extracts by HPLC. Protein content in the samples was between 0.4 and 0.6 mg/ml. Results are expressed as percentage of the control group. The mean±SEM of 5–8 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. *p<0.05; ***p<0.001.

3.3 Discussion

Adenosine, by activation of its four receptors, can modulate and influence several of the mechanisms that are dysregulated and implicated in the early stages of DR, in particular neurotransmission and inflammatory processes, where A_1R and $A_{2A}R$ exert a heavy influence (Wardas, 2002; Haskó et al., 2008). The results presented here provide clear evidence that the retinal adenosinergic system is affected by diabetes or hyperglycemic-like conditions. We observed that the levels of adenosine receptors are modified in retinas of diabetic rats and in retinal cells exposed to high glucose conditions, used to mimic the hyperglycemic conditions observed in diabetes. In particular, there was an up-regulation of A_1R and $A_{2A}R$, both in mRNA and protein levels (Vindeirinho et al., 2013), indicating that both high glucose conditions and the induction of diabetes are followed by alterations that may affect the adenosine signaling mechanisms. We also observed a decrease in the levels of ADA and AK in retinas four weeks after diabetes induction, but both of these enzymes remained unaltered in retinal cell cultures under HG. Furthermore, the activity of NTPDases was

skewed towards a higher rate of ATP/ADP degradation, agreeing with previous studies showing an increase in NTPDase1 levels.

There have been numerous descriptions of a general occurrence of adaptative changes to the density of adenosine receptors upon prolonged noxious conditions (Haskó et al., 2005). DR, being a prolonged condition that causes metabolic alterations to the cellular environment, can influence the adenosinergic system as well, although the mechanism by which streptozotocin-induced diabetes and high glucose conditions lead to the observed modification of the levels of retinal adenosine receptors still remains unclear, although more than one mechanism may be involved.

One of the possible motives for up-regulation of inhibitory A_1R in both HG and diabetic conditions may be the chronic inflammation environment created by hyperglycemic conditions in the retina. It has been shown in several reports that different agents and conditions can stimulate A_1R expression in an NF-kB-dependent manner (Nie et al., 1998; Hammond et al., 2004; Jhaveri et al., 2007). NF-kB, 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 in several cell types, including retinal cells (C. A. Aveleira et al., 2010), revealing a possible relationship between the high levels of TNF present in hyperglycemic conditions (Kern, 2007) and the increased levels of A_1R detected under the same conditions. Another possibility is the potential effect of the high levels of adenosine we registered in retinal cultures exposed to HG conditions: increased levels of adenosine coupled with increased levels of $A_{2A}R$ may indicate a potentiation of $A_{2A}R$ signaling, and a study performed in avian retinal cells showed a connection between high levels of $A_{2A}R$ activation and an up-regulation of A_1R, via a cAMP/PKA dependent pathway (Pereira et al., 2010). This upregulation was blocked when NF-kB inhibitors were used, indicating another link between NF-kB and A_1R regulation. The overall effect of A_1R is inhibitory, since its activation usually leads to a state of reduced activity, particularly in neurons where there is a decrease in neuronal excitability, firing rate and neurotransmitter release. The ability of this receptor 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 excessive release of glutamate is the cause of excitotoxicity, which leads to neuronal dysfunction and degeneration, and the retina is very susceptible to this phenomenon due to the fact that glutamate is the major excitatory neurotransmitter in the retina. It was observed an increase in glutamate release in retinal cultures exposed to high glucose and in retinas of diabetic animals (Santiago et al., 2006a), and this exposure of retinal cells to higher levels of glutamate can induce retinal cell death (Xin et al., 2007). The increase in A_1R levels in hyperglycemic conditions may have a neuroprotective effect, by down-regulating excessive excitatory neurotransmission and decreasing high Ca^{2+} influx levels, two features typical of diabetic conditions in the retina (Lieth et al., 1998; Kowluru et al., 2001; Pereira et al., 2010). This possible protective effect will be tested in Chapter 5.

The increased levels of $A_{2A}R$ observed in retinal cell cultures under HG and in diabetic retinas may also be due to the high levels of inflammatory markers found in those conditions. $A_{2A}R$ regulation is very sensitive to extracellular environment alterations, such as concentrations of inflammatory mediators (Haskó et al., 2008) and, in a similar manner to A_1R, $A_{2A}R$, can also be regulated by the transcription factor NF-kB (Murphree et al., 2005; Varani et al., 2009). $A_{2A}R$ can also be modulated by A_1R itself, with a study performed in mouse splenocytes showing that activation of A_1R can increase $A_{2A}R$ sensitivity (Naamani et al., 2014), which could be a result of increased $A_{2A}R$ levels (the study in question did not explore this possibility). However, since $A_{2A}R$ protein levels in diabetic retinas seem to increase earlier than A_1R (Vindeirinho et al., 2013) and are not sustained, this possibility can be discarded. In contrast with A_1R, $A_{2A}R$ has a more excitatory role in neurons, being responsible for the potentiation of synaptic transmitter release (Sebastião and Ribeiro, 2009), which raises the possibility that the increased levels of $A_{2A}R$ may be contributing to the neurotoxic environment that is prevalent in HG and diabetic conditions. Beyond excitotoxicity, $A_{2A}R$ may also

have a role in the damage inflicted by the inflammatory environment prevalent in the diabetic retina. Despite having a broad anti-inflammatory role in most immune cells (Haskó et al., 2008), there is evidence that $A_{2A}R$ has pro-inflammatory actions in microglia instead, controlling proliferation, NO levels, and even the release of pro-inflammatory cytokines (Santiago et al., 2014). The protective or damaging effects of $A_{2A}R$ in retinal cells in HG conditions, or in diabetic retinas, will be further explored in Chapter 5.

Although not visible in retinal cell cultures, A_3R showed increased expression levels one week after diabetes induction. A possible hypothesis for this alteration is, again, the inflammatory environment provided by diabetic conditions. A study made in rheumathoid arthritis models revealed an up-regulation of A_3R in human peripheral blood mononuclear cells caused by inflammatory cytokines, such as TNF and IL-2 (Madi et al., 2007). Additionally, these alterations were directly correlated to an increase in NFκB, and a blockade of IL-2 and TNF prevented the increase in both A_3R and NFκB. On the other hand, a study in BV2 microglial cells showed an A_3R-mediated inhibition of LPS-induced TNF expression that was associated with the inhibition of LPS-induced activation of PI3K/Akt and NFκB pathway (Lee et al., 2006), hinting at the possibility that A_3R may not only be regulated by TNF levels, but also regulate them in turn. After the registered increase at one week of diabetes, the levels of A_3R were decreased at the four weeks time point. It is possible, then that this transient increase in A_3R levels is part of a negative feedback mechanism triggered by the increased levels of pro-inflammatory cytokines, namely TNF, resulting from diabetic conditions. By the four weeks time point, the mechanism may be in the process of reverting A_3R density back to control levels as work in our lab revealed that, at eight weeks of diabetes, A_3R protein levels in diabetic retinas were the same as in control retinas (data not shown).

While the expression levels of A_3R showed an increase in the retinas of diabetic rats one week after the STZ injection, they were not altered in cell cultures under high

glucose conditions. This discrepancy in results may be due to humoral or neuronal signals that regulate A_3R levels *in vivo*, but are not present in *in vitro* conditions. Another cause may be due to the characteristics of our cell cultures: although our primary cell cultures possess all retinal cell types, their relative proportions may be different than what is found in the retina. If the increase in the A_3R observed in retinas is due to an effect in a particular cell type, this may not be reflected in our retinal cultures due to the differences in the proportions of cell types between cultures and retinas.

As a receptor with low affinity for adenosine, $A_{2B}R$ 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 (Feoktistov and Biaggioni, 1997; Kreckler et al., 2006; Haskó et al., 2008). In other circumstances their role is more supportive of $A_{2A}R$, becoming active in situations where $A_{2A}R$ can become desensitized (Ralevic and Burnstock, 1998). There were no alterations observed in both retinal cell cultures under HG conditions and diabetic retinas, leading us to question if $A_{2B}R$ is regulated by other means beyond an increased gene expression or protein translation, or if it is simply unaffected by the environment of the diabetic retina. There is some evidence that TNF can increase $A_{2B}R$ activity without increasing mRNA or protein levels (Trincavelli et al., 2004), possibly by inhibiting receptor desensitization, so it is possible that $A_{2B}R$ can still be affected in DR.

The bi-directional nucleoside transporters provide a pivot point in the regulation of both intra and extracellular levels of adenosine. Equilibrative transporters will function according to the gradient, allowing extra and intracellular mechanisms to influence one another, while the concentrative transporters can actively transport adenosine against the gradient, offering a mode of circumventing those mechanisms. The increased levels of ENT1 in retinal cultures under HG may be a response of the system to the

discrepancy of concentrations of adenosine between the extracellular and intracellular space. It has been shown in another cellular model, aortic smooth muscle cells, that high levels of glucose can increase the expression of ENT1 (Leung et al., 2005), and this seems to be via MAPK/ERK-dependent pathways. However, the regulation of nucleoside transporters seems to be variable between tissues and even cell types, for another study showed that ENT1 expression is decreased in the heart and liver of diabetic rats (Pawelczyk et al., 2003). In diabetic retina, only the CNT2 transporter showed alterations, with an increase at four weeks of diabetes. Like the equilibrative transporters, the effect of diabetes on the levels of concentrative transporters also seems to vary according to the tissue and cell type observed (Pawelczyk et al., 2003; Sakowicz et al., 2004; Rodríguez-Mulero et al., 2005). As mentioned before, the difference in ENT1 levels between retinal cultures under HG conditions and diabetic retinas may be due to the inherent differences between a primary cell culture model and the tissue itself, or the proportion of cell types present in the cultures, which can oscillate and affect the variability of the results. As for the levels of CNT2, is it also possible that seven days of HG exposure may not be enough to induce alterations.

ADA and AK are key elements responsible for the regulation of intracellular and extracellular adenosine, by phosphorylation to AMP by AK or deamination to inosine by ADA. The decrease observed in the levels of both ADA and AK in diabetic retinas 4 weeks after diabetes induction may signal a severe dysregulation of adenosine levels in diabetic conditions, since it has been shown that a blockade of the activity of ADA and AK lead to a massive increase in adenosine concentration in various tissues (Ely et al., 1992; Pak et al., 1994; Chunn et al., 2006).

The enzymes responsible for the degradation chain of ATP/ADP/AMP are vital for the management of both extracellular ATP and adenosine levels, often becoming the steps connecting the different signaling systems and allowing coordination between them (Haskó et al., 2008; Wurm et al., 2008). In these results, it was shown that the activity of NTPDases was increased, in degradation of both ATP and ADP. A study performed

in the same retinal culture model revealed an increase in depolarization-evoked ATP release, and a parallel impairment in ATP degradation in the same conditions, suggesting that ATP signaling mechanisms are sustained for longer in hyperglycemic conditions (Costa et al., 2009). This would seem to be in contradiction with the results shown here. However, the fact that most NTPDases, including NTPDase1 and 2, degrade ATP means we cannot differentiate their actions when incubating cells with ATP. Furthermore, since the enzymatic assay quantifies the total released Pi, it can be affected by downstream degradation of ADP during the incubation time of the assay. On the other hand, the increase in ADP degradation observed in HG conditions can be linked to the reported increase in expression levels of NTPDase1 (Wurm et al., 2008). This work of Wurm *et al.* showed that the distribution and expression levels of retinal NTPDase1 were altered and increased in diabetic conditions, and this enzyme degrades both ATP and ADP fairly well. This, predictably, would have an impact on the ability of retinal cells to degrade ADP, and ATP to a lesser extent. It is possible, then, that the mechanisms behind the delay in ATP degradation observed in the study of Costa *et al.* are being masked here by the effects of NTPDase1, since the experimental methods used were different, with Costa *et al.* using the more direct ATP quantification method with the luciferin-luciferase assay. Unlike ATP/ADP, the degradation of AMP was not affected by HG conditions in retinal cells, which means 5'-NT's activity remains at normal levels. Still, the increased availability of AMP due to an increased degradation of ATP/ADP has probably contributed to the increased extracellular adenosine concentrations observed in retinal cultures under HG conditions, along with the previously described decrease in ADA activity in the same conditions (Vindeirinho et al., 2013). Such an increase in adenosine concentration may affect the signaling carried out by the adenosine receptors, particularly $A_{2B}R$, the receptors with lower affinity for adenosine, possibly contributing to an attempt at combating the prevalent inflammatory environment, since this receptor is also recognized as an important mediator of inflammation (Haskó et al., 2008).

Taken together, these results demonstrate that the retinal adenosinergic system is affected by diabetes and high glucose conditions. The alterations observed in the expression levels of the receptors A_1R, $A_{2A}R$ and A_3R, of the transporters ENT1 and CNT2, enzymes AK and ADA, as well as the impact of NTPDase activity and extracellular adenosine levels, can have a significant impact on the inflammatory and excitotoxic conditions observed in diabetic retinas. How the modulation of this system can prevent or exacerbate this will be explored further in Chapter 5.

Chapter 4 Interactions between ADA and DPP4 in high glucose conditions

4.1 Introduction

As mentioned before in Chapter 1, the mechanism of adenosine removal by ADA is one of particular importance in disruptive situations. Most disturbances in homeostasis are followed by a surge in extracellular adenosine levels: pH shifts (Cheng et al., 2000), infection (Tonin et al., 2014), and hypoxia (Phillis et al., 1992; Saito et al., 1999) are just a few examples. This increase can be linked in most occasions to a protective effect of adenosine, mainly through neuromodulatory (Wardas, 2002) and anti-inflammatory actions (Eltzschig et al., 2004). However, chronically high levels of adenosine observed in disruptive situations, such as ischemia or inflammation, can be damaging (Ghiardi et al., 1999). In those situations ADA activity rises (Eltzschig et al., 2006; Pimentel et al., 2009), playing a crucial role in adenosine removal, in a feedback mechanism seemingly designed to prevent the toxic effects of prolonged adenosine exposure (Haskó and Cronstein, 2004). This is evidenced by the fact that ADA-deficient mice develop chronic lung damage associated with high pulmonary levels of adenosine (Blackburn, 2003). ADA does not have its own hydrophobic/transmembrane domain, so it requires alternative anchoring mechanisms to exert its functions at the cell surface level. For that it can bind to a number of other proteins that will function as anchors, such as CD26 (Dong et al., 1996), A_1R (Torvinen et al., 2002), and $A_{2B}R$ (Antonioli et al., 2014).

The previously described decrease in ADA activity under HG conditions (Vindeirinho et al., 2013) can be a result of alterations to these anchoring mechanisms, or a direct influence of the anchor proteins on the activity of the enzyme. One hypothesis may be the interaction between ADA and CD26. CD26, also called adenosine deaminase complexing protein 2 or DPP4, is a multi-functional cell surface peptidase involved in glucose and insulin regulation mechanisms, and selective DPP4 inhibitors are available as treatment for diabetes type 2 (Green et al., 2006), exerting its actions mainly through the prolonged insulinotrophic effects of GLP-1, one of DPP4's numerous

substrates (Mulvihill and Drucker, 2014). Beyond being present at the cell surface level, DPP4 can be cleaved into a smaller soluble form that can travel the bloodstream (Drucker, 2007), and also has enzymatic activity. DPP4 exhibits a complex mechanism of action: beyond its peptidase activity, it possesses a small intracellular tail that is able to activate intracellular signaling pathways, and beyond that, is also able to interact with several ligands, of which ADA is one (Zhong et al., 2013b). The DPP4-ADA binding complex is able to regulate several pathways, especially in inflammatory situations, not just by indirect effects of adenosine, but also by a co-stimulatory action (Zhong et al., 2013b). In fact, DPP4 levels can rise in parallel with ADA in noxious conditions, such as hypoxia (Eltzschig et al., 2006), contributing to the reduction of adenosine levels. All of this makes DPP4 a potential player in the alterations occurring to ADA activity levels in retinal cultures under HG conditions. Therefore, we set out to study the protein levels of this peptidase in retinal cell cultures and diabetic retinas, and ascertain the effects of DPP4 and DPP4 inhibitors on ADA protein and activity levels.

4.2 Results

4.2.1 Diabetes/high glucose does not alter the protein levels of DPP4 in retinal cell cultures and diabetic rat retinas

The presence of DPP4 in retinal cell cultures and in retinas was confirmed by western-blot. As illustrated in Figure 20A, there were no alterations to the protein levels of DPP4 in retinal cells cultured in HG conditions, or with the osmotic control, when compared to control conditions (107.2±3.15% of control and 92.45±1.59%, respectively). Similar results were observed in diabetic retinas where, despite a tendency for increase after one week of diabetes, as see in Figure 20C, DPP4 levels had no significant alterations in response to diabetic conditions, neither at one

(120.7±20.61% of control), nor at four weeks after diabetes induction (91.4±9.36% of control).

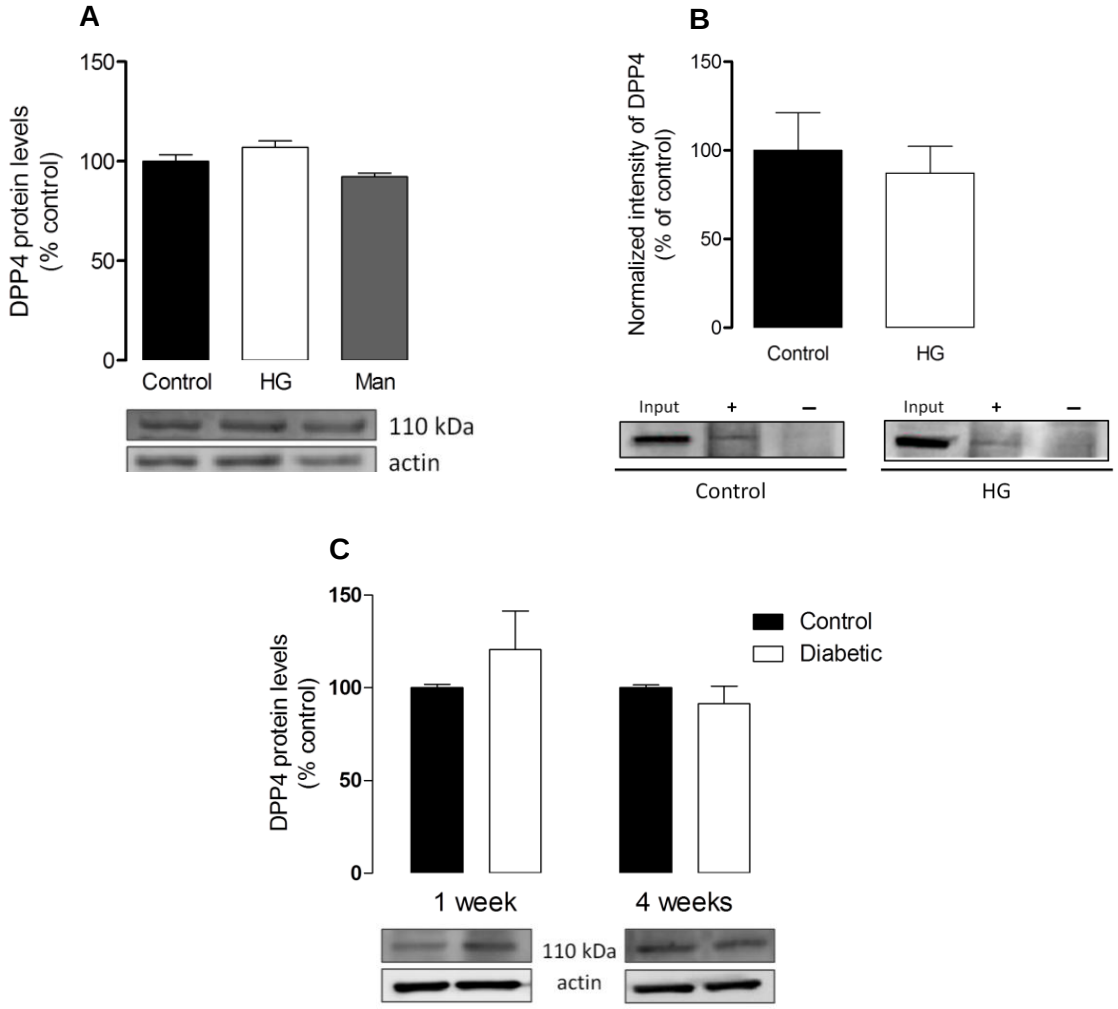

Figure 20: DPP4 protein levels in cultured retinal cells and diabetic retinas. (A) The protein levels of DPP4 were evaluated by Western Blot analysis, seven days after HG incubation. The mean±SEM of 3–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. **(B)** Protein extracts were subjected to immunoprecipitation with ADA as target, as described in Chapter 2. The eluates from the beads were analyzed by Western Blot for DPP4. A portion of the sample not subjected to the procedure was used as input, and the negative control had the corresponding IgG, instead of α-ADA antibody. The mean±SEM of 4 independent experiments was analyzed with the Student's t-test (HG vs. control) and F test. **(C)** The protein levels of DPP4 were also evaluated by Western Blot analysis, one and four weeks after diabetes induction. The mean±SEM of 3-5 independent experiments was analyzed with the Student's t-test (diabetic vs. control) and F test.

Beyond assessing the levels of DPP4 in HG conditions, the interaction between the peptidase and ADA, specifically the formation of the binding complex ADA-DPP4, was of particular relevance. To assess this, we used retinal cell cultures exposed to HG for seven days and performed a co-immunoprecipitation assay (Co-IP). We incubated the extracts with a α-ADA antibody and, after precipitation, we performed a western blot to determine if DPP4 had been co-precipitated with ADA. Due to the demands of the procedure, we did not use mannitol, for a more efficient use of sample material. As seen in Figure 20B, DPP4 was co-precipitated with ADA, showing evidence to support an ADA-DPP4 interaction in primary rat retinal cell cultures. Although the amount of DPP4 co-precipitated had a tendency to decrease in HG conditions, this result was not significant (87.25±15.02% of control).

4.2.2 Soluble DPP4 increases ADA activity and protein levels in retinal cell cultures under HG conditions

To evaluate the effects that DPP4 may have in HG conditions on the activity of ADA, we used a commercially available soluble form of DPP4 that is able to bind to ADA, and has been used for competitive binding assays (Zhong et al., 2013a). We measured ADA activity in retinal cell cultures, after seven days of HG exposure, by incubating the samples with adenosine and measuring the amount of ammonia released. Cells were incubated with DPP4 (5μg/ml) at the fifth day of HG exposure (48h incubation period), and one hour before the activity was measured. We have previously shown that ADA activity is not affected by the osmotic control in HG conditions (Vindeirinho et al., 2013) and as such, we did not use mannitol for this set of experiments. Figure 21A shows that, as described in our previous work, ADA activity was severely decreased in retinal cells exposed to HG conditions (35.89±1.41% of control, $p<0.001$). Incubation of retinal cells with DPP4 for one hour did not revert the decrease in ADA activity, remaining at 55.26±14.76% of control ($p<0.01$). However,

when cells were incubated with DPP4 for 48 hours before the enzymatic assay, the enzymatic activity of ADA dramatically increased in HG conditions, rising up to 164.3±14.52% of control ($p<0.001$). In both incubation time points (one and 48 hours) DPP4 had no relevant effect on ADA activity in control conditions.

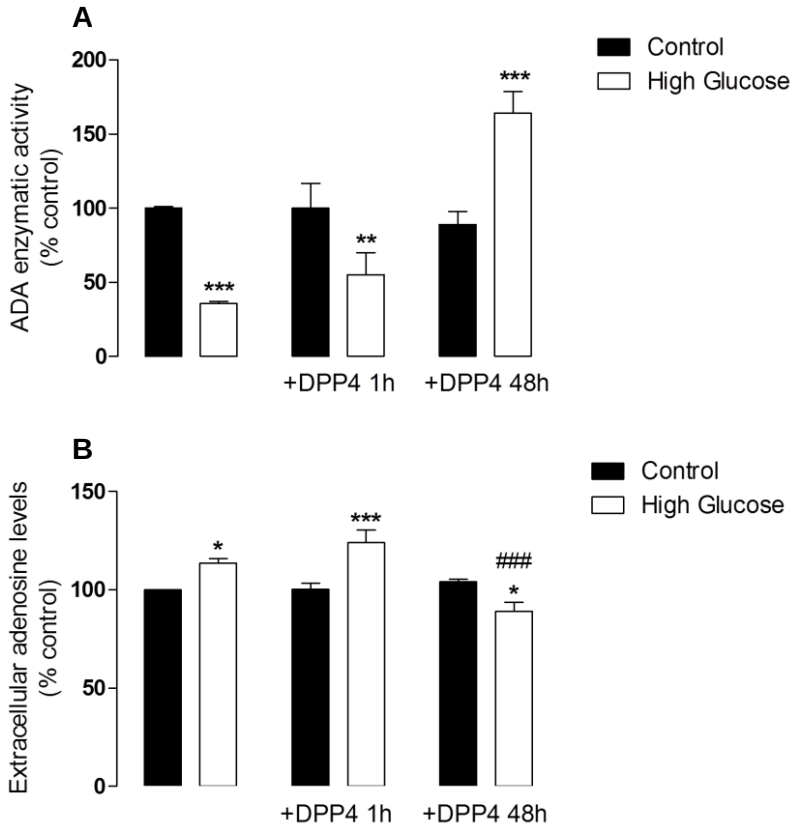

Figure 21: Effect of DPP4 incubation on the activity of ADA and adenosine levels in retinal cell cultures under HG. ADA activity and adenosine levels were analyzed in retinal cells after seven days of HG exposure. Soluble DPP4 (5µg/ml) was added 48h or 1h before the experiment. **(A)** The activity of ADA was measured by incubating the samples with adenosine and assessing the amount of ammonia produced, after normalization with the sample's protein content. The mean±SEM of 3-5 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. **p<0.01; ***p<0.001. **(B)** The levels of adenosine were measured in 20 µl of culture medium by HPLC and normalized for the protein content. Results are expressed as percentage of the control group. The mean±SEM of 5–7 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. *p<0.05; ***p<0.001; ### p<0.001 in relation to HG+DPP4 1h.

ADA activity can directly affect extracellular adenosine levels, and its low activity in HG conditions may contribute to the increased extracellular adenosine concentration observed in HG conditions (Chapter 3). Therefore we evaluated the adenosine concentrations found in culture medium of retinal cell cultures under HG conditions, after incubation with DPP4. As illustrated in Figure 21B, adenosine levels were again significantly higher in HG conditions when compared to control conditions (113.5±2.39% of control, $p<0.05$), and they remained high after one hour of DPP4 incubation, even increasing surprisingly to 124.1±6.38% of control ($p<0.001$). Conversely, when cells were incubated with DPP4 for 48 hours, the extracellular levels of adenosine measured in retinal cells cultured in HG conditions were below control levels (88.95±4.60% of control, $p<0.05$). This is in accordance with the increased ADA activity recorded in the same conditions, and raises the possibility that, due to the different time points, incubation with DPP4 may not be having a direct effect on ADA activity, but may be increasing the activity through indirect routes. To test this hypothesis, we evaluated the protein levels of ADA after 48 hours of DPP4 incubation.

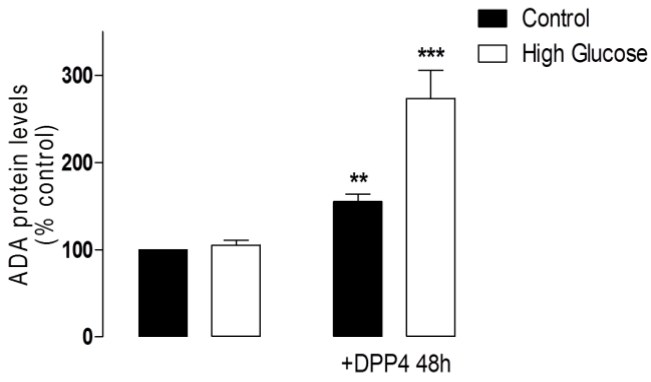

Figure 22: Effect of DPP4 incubation on ADA protein levels in cultured retinal cells under HG. The protein levels of ADA were evaluated by Western Blot analysis, seven days after HG exposure. Soluble DPP4 (5µg/ml) was added 48h before the experiment. The mean±SEM of 3–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test.

As seen in Figure 22, 48 hours of DPP4 incubation increased the protein levels of ADA in control conditions (155.1±8.24% of control, $p<0.01$), and had an even greater impact in HG conditions, with levels rising to 273.4±32.37% of control ($p<0.001$). This may explain then, the increased activity of ADA in HG conditions after 48 hours of DPP4 incubation.

4.2.3 Inhibition of DPP4 enzymatic activity partially blocks DPP4 incubation effect, but does not affect a possible endogenous DPP4 modulation of ADA in retinal cell cultures under HG conditions

DPP4 can exert modulatory effects through interaction with other proteins, by activation of signaling pathways, but mainly through its enzymatic activity, by controlling concentrations of its numerous substrates. To better understand how DPP4 was exerting its modulatory effects on ADA activity and protein levels, we incubated retinal cell cultures with vildagliptin, a DPP4 inhibitor that is commercially available as an effective oral antidiabetic agent (Mathieu and Degrande, 2008). We used retinal cell cultures exposed to HG conditions and pre-incubated the cells with vildagliptin (2nM) two minutes before incubation with DPP4, or kept the cells incubated with vildagliptin alone. After 48 hours we assessed the levels of ADA activity, protein density and extracellular adenosine concentrations.

Figure 23A shows that treatment with vildagliptin managed to only partially block the effect of DPP4 incubation in HG conditions, reducing the increased activity levels of ADA induced by DPP4, from 164.0±24.16% of control ($p<0.001$) to 144.3±17.19% but not enough to push them below control levels, as seen when cells under HG conditions had no treatment. Vildagliptin treatment alone, on the other hand, seemed to have no significant effect on the low levels of ADA activity levels in HG conditions (43.56±19.92% of control, $p<0.01$).

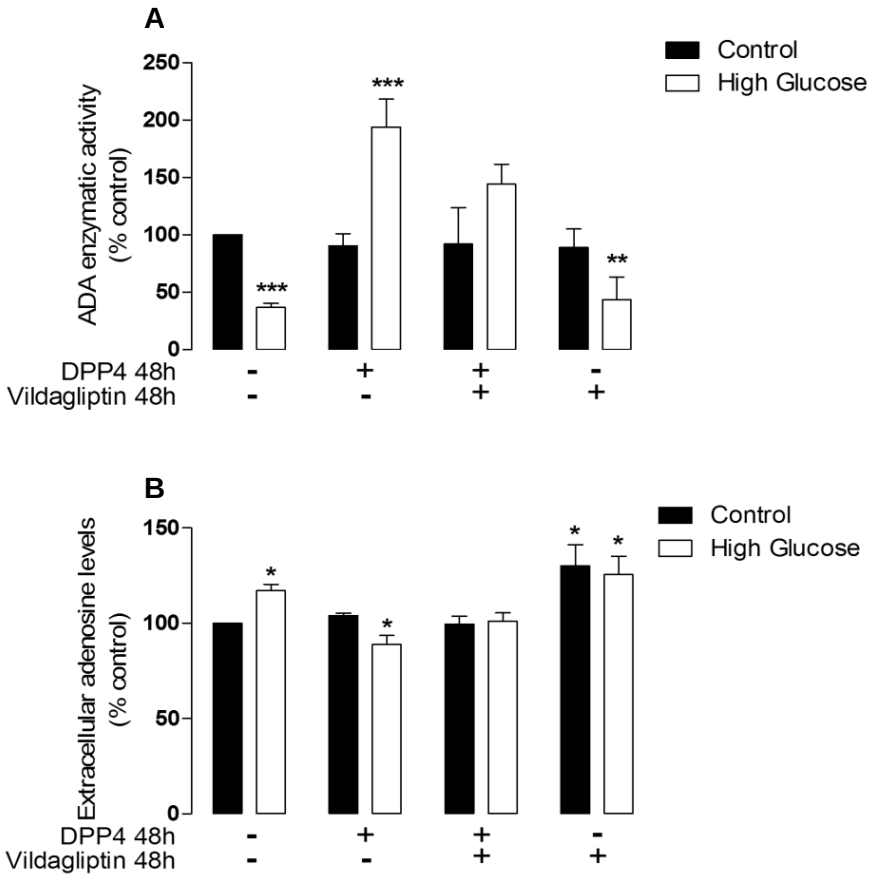

Figure 23: Effect of DPP4 and Vildagliptin incubation on the activity of ADA and adenosine levels in retinal cell cultures under HG. ADA activity and adenosine levels were analyzed in retinal cells after seven days of HG exposure. Soluble DPP4 (5µg/ml) and vildagliptin (2 nM) were added 48h before the experiment. **(A)** The activity of ADA was measured by incubating the samples with adenosine and assessing the amount of ammonia produced, after normalization with the sample's protein content. The mean±SEM of 4-7 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. **p<0.01; ***p<0.001. **(B)** The levels of adenosine were measured in 20 µl of culture medium by HPLC and normalized for the protein content. Results are expressed as percentage of the control group. The mean±SEM of 4–7 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. *p<0.05.

As for extracellular adenosine concentrations, again vildagliptin induced a partial inhibition of DPP4 action in HG conditions (Figure 23B), recovering the lower levels of adenosine induced by DPP4 incubation from 88.95±4.60% of control (p<0.05) to control levels (101.0±4.38% of control), but still not enough to allow the increase typical

of HG conditions. When cells were incubated with vildagliptin alone, however, adenosine levels remained high in HG conditions (125.5±9.55% of control, $p<0.05$). More surprisingly, adenosine levels were also significantly increased in control conditions, when vildagliptin was used (130.2±10.95% of control, $p<0.05$).

Despite only partially inhibiting the actions of DPP4 on ADA activity and adenosine levels, vildagliptin was able to fully inhibit DPP4 induction of ADA protein levels (Figure 24), reverting the increase induced in both control and HG conditions (155.1±8.24% and 273.4±32.37% of control respectively, $p<0.01$ and $p<0.001$) back to levels not significantly different from control (112.5±5.06% and 111.3±6.97% of control, respectively). As for vildagliptin treatment alone, it had no discernible effect on ADA protein levels, on both control and HG conditions (98.22±6.43% and 98.63±7.04% of control, respectively).

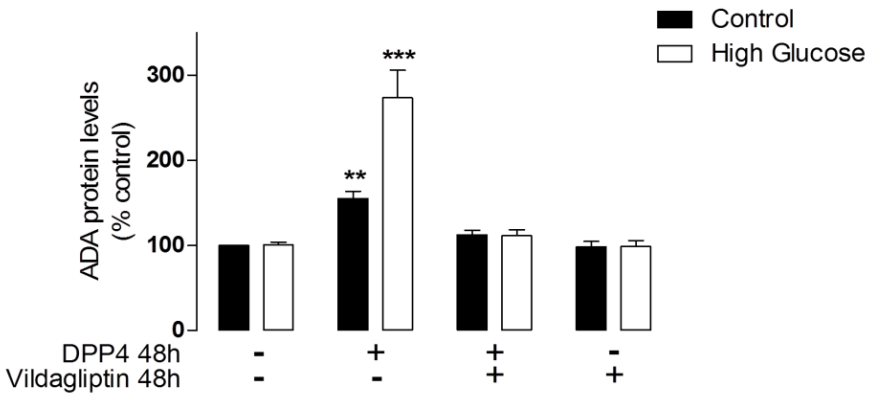

Figure 24: Effect of DPP4 and Vildagliptin incubation on ADA protein levels in cultured retinal cells under HG. The protein levels of ADA were evaluated by Western Blot analysis, seven days after HG exposure. Soluble DPP4 (5µg/ml) and vildagliptin (2 nM) were added 48h before the experiment. The mean±SEM of 3–5 independent experiments was analyzed with one-way ANOVA test and Tukey's multiple comparison test. **p<0.01; ***p<0.001.

4.3 Discussion

When addressing a possible influence of anchoring proteins on the reduced activity of ADA in retinal cell cultures under HG conditions, DPP4 was an interesting target because it is not only involved in glucose and insulin regulation, but also in inflammatory processes (Dobrian et al., 2011; Wronkowitz et al., 2014), both through its hydrolysis of numerous regulatory peptides, neuropeptides and chemokines, but also by co-stimulatory processes with other proteins such as CXCR4, CD45 and ADA (Mulvihill and Drucker, 2014).

Because DPP4 levels can accompany ADA levels of activity (Eltzschig et al., 2006), we sought to know if the same was happening in our retinal cell cultures in HG conditions. The protein levels of DPP4 were unaltered in both HG conditions and in the retina of diabetic rats, one and four weeks after diabetes induction, but the amount of DPP4 co-precipitated with ADA in retinal cell cultures showed a strong tendency to decrease in HG conditions, which could ultimately affect ADA activity levels. This result by itself is surprising, since it is known that rodent DPP4 does not bind to ADA (Weihofen et al., 2004), due to residues located in the cysteine-rich domain of DPP4 being different from those of human DPP4, although we are not the first to report co-immunoprecipitation of DPP4 with ADA in rats (Kröller-Schön et al., 2012). It is possible that soluble DPP4 can interact with ADA without forming the binding complex, in a lighter or more indirect connection that can be responsible for the co-precipitation of DPP4 alongside ADA in rat retinal cell cultures, and a more powerful experimental approach would need to be used to explore this result. As for the variability observed in co-immunoprecipitation assays, responsible for the high error, it may be due to the inherent diversity within cell populations characteristic of our primary mixed retinal cell cultures, and puts forward the possibility that this connection is more prominent or even restricted to one or more particular cell types.

When a soluble form of DPP4 was added to our cultures for 48 hours, a strong increase in ADA activity under HG conditions was observed, also resulting in lower extracellular adenosine levels. This increase in activity seems to be due to an increase in overall ADA protein levels, which DPP4 incubation managed to increase even in control conditions, although without a discernible rise in activity.

When cells were pre-incubated with vildagliptin this protein increase was abolished, showing a strong possibility that the mechanisms by which DPP4 increased ADA protein levels depend on its enzymatic activity, either by non-enzymatic actions that may still require an active catalytic site (Ohnuma et al., 2001; Ikushima et al., 2002), or by direct influence of DPP4 substrates that may influence the levels of ADA. The difference in DPP4 incubation results in control and HG conditions may indicate that the possible substrates in play are also affected by HG conditions, which would explain why the effects of DPP4 incubation were more visible and effective. These substrates may be ones that can downregulate ADA transcription or translocation to the cell membrane, and their truncation by DPP4 would cause that inhibition to decrease. One possible example may be the vasoactive intestinal peptide (VIP), a neuropeptide with anti-inflammatory and also anti-hyperglycemic effects (Onoue et al., 2008; R. Yu et al., 2011), which is present in the retina (Casini et al., 1994). Since the decrease of ADA activity is often used as a marker of anti-inflammatory action (Pereira et al., 2006; Fröde et al., 2009), it is possible that VIP may have a down-regulatory effect on ADA and that the increased action of DPP4 blocks such effect by truncating the active form of VIP. Furthermore, VIP has been shown to be affected in diabetic conditions (Belai et al., 1993; Shotton et al., 2004), which could also explain the stronger effects of DPP4 incubation observed in HG conditions. It would be interesting to assess VIP levels in retinal cultures under HG conditions and in the diabetic retina, and to further explore this possible interaction. Apart from inactivation, several DPP4 substrates gain different functions in their cleaved forms after DPP4 action. One such example is neuropeptide

Y (NPY), which is N-terminally truncated to NPY (3-36), loses its ability to bind to the Y1 receptor and becomes an Y2/Y5 receptor selective agonist (Mentlein et al., 1993).

The increase in activity of ADA, however, was only partially blocked, showing that DPP4 was affecting the activity of ADA not just by increasing protein levels, but by more than one mechanism. This other mechanism may be the interaction shown by the co-immunoprecipitation. It seems to be independent of DPP4 enzymatic activity, and could account for the recovery of control adenosine levels in HG conditions, when DPP4 is added after vildagliptin. Beyond its peptidase activity, soluble DPP4 could be inducing an increased activity of ADA, by influence of other DPP4 ligands, by increasing the number of DPP4 homodimers at the cell surface and increasing the signaling mechanisms activated by the intracellular tail of DPP4, or by direct signaling mediated by the soluble DPP4.

Treatment with vildagliptin alone, on the other hand, showed no effect apart from raising the extracellular adenosine levels in both control and HG conditions, pointing to the conclusion that endogenous DPP4 is not affecting ADA protein or activity levels, or at least not affecting enough to have a physiological impact on ADA. Only when levels are increased by the supply of soluble DPP4 do we witness a marked influence of DPP4 in ADA activity and protein levels in HG conditions, showing that it is probable that endogenous DPP4 levels may be too low for a proper influence on ADA activity. One other hypothesis would be that the observed DPP4 incubation effect is a soluble DPP4 action only, since soluble DPP4 is also able to activate intracellular signaling pathways, in a manner either requiring catalytic activity or even being independent from it, and not needing ADA binding (Ohnuma et al., 2001; Ikushima et al., 2002; D. M. T. Yu et al., 2011). If so, then an assessment of soluble DPP4 in rat retinal cell cultures would be interesting to understand why retinal cell cultures were not affected by vildagliptin incubation. The mechanisms underlying the actions of soluble DPP4, however, are still poorly understood (Zhong et al., 2013b).

Chapter 5 Modulation of adenosine receptor signaling, and its impact in HG conditions/diabetic retina

5.1 Introduction

As mentioned in Chapter 1, the early stages of DR are characterized by a neurodegenerative environment, with increased levels of excitotoxicity, altered signaling pathways, and chronic inflammation, all leading to increased cell death, morphological changes and loss of retinal function (Antonetti et al., 2006).

It is very likely that the different ARs may be involved in this toxic environment, and the alterations observed in the levels of A_1R and $A_{2A}R$ in HG conditions and in diabetic retinas can be a direct result of one of more of these processes, either exacerbating the situation, or in a response against the loss of homeostasis. However, AR signaling is a complex system, and the actions of these receptors can vary from protective to disruptive according to the concentration of adenosine available, the time point, type of tissue, cell type, environment, and their actions can also shift over time in chronic conditions (Wardas, 2002; Socodato et al., 2011; Sheth et al., 2014). The increase in A_1R levels in hyperglycemic conditions may help control the excessive excitatory neurotransmission and osmotic swelling (Uckermann et al., 2006). However A_1R can also mediate pro-inflammatory actions, resulting in a negative effect (Cronstein et al., 1992; Schnurr et al., 2004). On the other hand, $A_{2A}R$ activity can exacerbate the neurotoxic environment by increasing excitatory transmitter release, and its effect on inflammation and angiogenesis can also be disruptive (Takagi et al., 1996; Popoli et al., 2002; Gyoneva et al., 2014). Even adenosine itself can switch from protective to toxic in prolonged noxious conditions if the levels remain high, due to the formation of superoxide radicals after adenosine degradation (Ghiardi et al., 1999).

Therefore we set out to evaluate the effects of the modulation of A_1R and $A_{2A}R$ activity on cell death in retinal cell cultures under HG conditions, by using selective agonists and antagonists. We also evaluated the effects of an increase in extracellular adenosine concentration, by blocking ENT1.

Afterwards, we chose the most promising method, and applied it to *in vivo* studies, using diabetic rats, to assess the effects not only on retinal cell death, by monitoring retinal ganglion cell (RGC) survival, but also on retinal function, by performing ERG analysis.

5.1.1 The Electroretinogram

An ERG is the recording of extracellular electric currents generated by retinal cells in response to a light stimulus. It is recorded at the surface of the cornea, and it is generated by radial currents stemming directly from retinal neurons or indirectly from the changes in extracellular potassium levels that neuronal activity causes, that then affect retinal glial cells (Rosolen et al., 2008). Because of this, the ERG is the summed activity of all cells in the retina, and the impact of the different populations of retinal cells is discerned by understanding their respective contribution to the ERG wave.

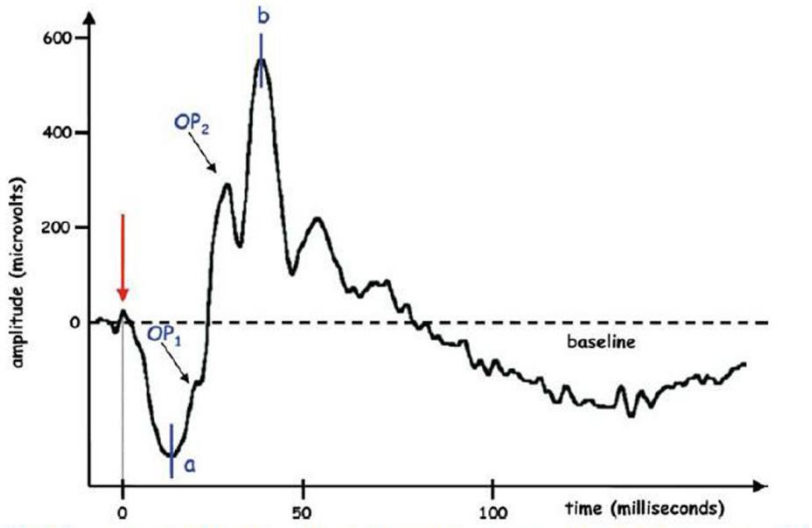

Figure 25: Representation of a flash scotopic electroretinogram. An initial negative a-wave is followed by a positive b-wave. Oscillatory potentials (OPs) are wavelets that appear in the rising phase of b-wave. (in Rosolen et al., 2005)

The most common focus of ERG recording is on the negative a-wave and the positive b-wave (Figure 25), as well as the oscillatory potentials (OPs), which are components of high frequency and low voltage usually present on the rise of the b-wave (Marmor et al., 2004). The a-wave is the first major component of the ERG, and is the result of photoreceptor responses for the most part, although post-receptoral contributions can influence the a-wave, especially at lower light intensities (Bush and Sieving, 1994). The positive b-wave is the largest component of the flash ERG, and is largely the result of bipolar cell responses, although there is some evidence to support a role for Müller cells in contributing to the b-wave alongside (Rosolen et al., 2008). The b-wave recorded in low light intensity, or scotopic, conditions is attributed to the ON-bipolar cells, while in high light intensity, or photopic, conditions there seems to be a concerted effort between ON and OFF-bipolar cells to shape the rise and fall of the b-wave (Sieving et al., 1994). The OPs can vary in number according to stimulus intensity, inter-stimulus interval and retinal adaptation, and are also sensitive to pharmacological manipulation and pathological conditions (Rosolen et al., 2008). They reflect neuronal synaptic activity from a variety of neuronal cell types, and dopamine-, GABA-, and glycine-mediated neuronal pathways can be involved in the generation of OPs (Wachtmeister, 1998).

Beyond the a-wave, b-wave and OPs, flicker responses are also recorded. A stimulus with a frequency higher than 5 Hz elicits a photoreceptor-driven response, since only they are able to follow rapid changes in luminance and respond to them using circuits than differ from the circuits used in stimuli with lower frequencies (Baron et al., 1979).

The ERG is a very useful, non-invasive technique that provides us with numerous parameters to assess the state of different cell populations in the retina. However, it is not without its limitations, especially when dealing with rats and DR. The low number of cone photoreceptors of rat retina means that the a-wave is lost in a normal photopic recording. As for DR, while some works showed that as soon as two weeks of diabetes there are alterations occurring, mainly in OP latencies and amplitudes (Sakai et al.,

1995; Li et al., 2002), others only see alterations after six, eight or ten weeks, again in OPs but also in b-wave latencies and amplitudes (Hancock and Kraft, 2004; Kohzaki et al., 2008; Pardue et al., 2014). Some of these discrepancies may be explained by differences in animal model of DR, time-point, or experimental procedures.

5.2 Results

5.2.1 *In vitro* modulation of A_1R and $A_{2A}R$ signaling, and ENT1 blockade

To evaluate the effects that A_1R and $A_{2A}R$ signaling, as well as ENT1 blockade could have on retinal cell survival, we used selective agonists and antagonists of the receptors and an ENT1 blocker. Since (Costa et al., 2012) have shown that retinal cells start to lose viability at the third day of exposure to HG conditions, we used that time point, and started the incubation with the agonists or antagonists at the third day of HG exposure, with a reinforcement at the fifth day.

5.2.1.1 A_1R agonist decreases cell death in retinal cell cultures under HG conditions

To evaluate the effects that A_1R signaling activation or inhibition may have on cell survival, we incubated the cells with a selective agonist, 2'-MeCCPA (50 nM), or a selective antagonist, DPCPX (50 nM), twice over the last four days of HG exposure. Afterwards, cells were incubated with PI, a fluorescent dye that binds DNA. Since PI is membrane impermeable, it is excluded from viable cells and only stains the nucleus of late apoptotic, necroptotic and necrotic cells (Lecoeur, 2002). As seen in Figure 26, HG conditions significantly increased the number of PI-positive cells (160.9±5.57% of control, $p<0.01$). Blockade of A_1R with DPCPX had no relevant effect on cell survival either in control or in HG conditions [101.3±4.16% and 156.5±17.38% of control

(p<0.01), respectively]. Incubation with the A_1R agonist, on the other hand, was able to significantly reduce the number of PI-positive cells observed in retinal cell cultured in HG conditions, down to 111.5±4.37% of control, but had no significant effect on cell death in control conditions.

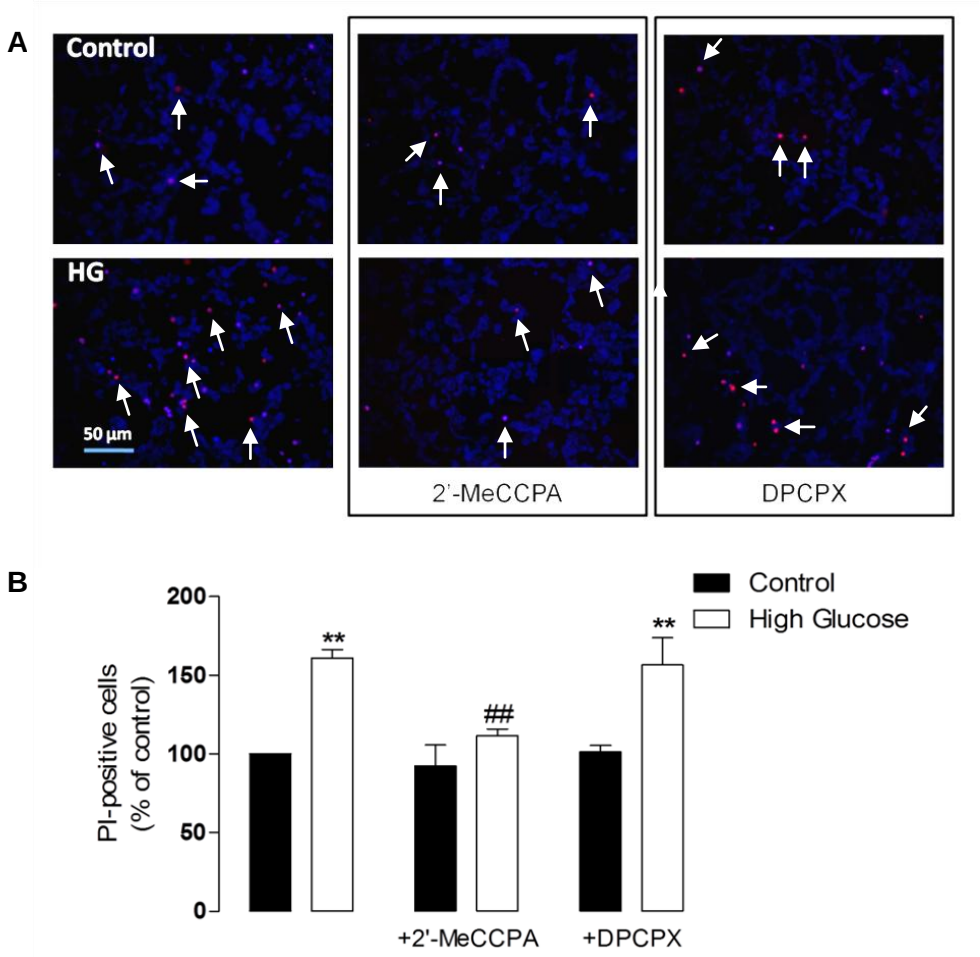

Figure 26: Effect of A_1R modulation on cell death in retinal cell cultures under HG conditions. Cell death was evaluated in retinal cells after seven days of HG exposure by the PI method. The A_1R selective agonist 2'-MeCCPA (50 nM) or the selective antagonist DPCPX (50 nM) were added twice over a span of four days before PI incubation. **(A)** Hoechst 33342-stained nuclei appear in blue, PI appears in red. Arrows denote dying cells, where PI and Hoechst 33342 co-localize at the nucleus. **(B)** Results are expressed as percentage of the control group. The mean±SEM of 3-5 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. **p<0.01; ##p<0.01 in relation to HG.

5.2.1.2 $A_{2A}R$ antagonist decreases cell death in retinal cell cultures under HG conditions

To assess the effects that $A_{2A}R$ signaling activation or inhibition may have on cell survival, we incubated the cells with a selective agonist, CGS 21680 (30 nM), or a selective antagonist, SCH 58261 (50 nM), twice over the last four days of HG exposure, followed lastly by PI incubation to determine cell death.

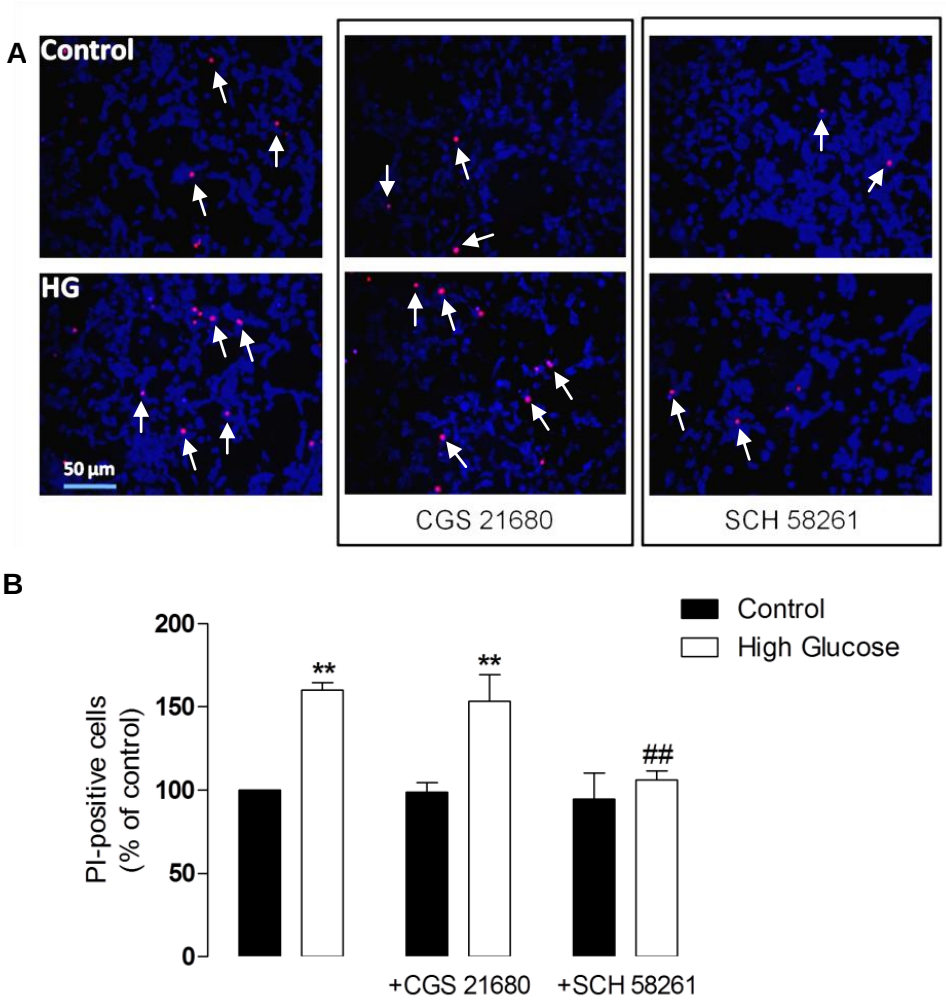

Figure 27: Effect of $A_{2A}R$ modulation on cell death in retinal cell cultures under HG conditions. Cell death was evaluated in retinal cells after seven days of HG exposure by the PI method. The $A_{2A}R$ selective agonist CGS 21680 (30 nM) or the selective antagonist SCH 58261 (50 nM) were added twice over a span of four days before PI incubation. **(A)** Hoechst 33342-stained nuclei appear in blue, PI appears in red. Arrows denote dying cells, where PI and Hoechst 33342 co-localize at the nucleus. **(B)** Results are expressed as percentage of the control group. The mean±SEM of 4-5 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. **p<0.01; ##p<0.01 in relation to HG.

The number of PI-positive cells was again significantly increased in HG conditions, as seen in Figure 27 (160.2±4.15% of control, $p<0.01$), and treatment with the $A_{2A}R$ agonist was unable to affect it (153.2±16.11% of control, $p<0.01$). Conversely, when cells were incubated with the $A_{2A}R$ antagonist, there was a marked reduction in the number of PI-positive cells in HG conditions, attaining a value close to that observed in control conditions (106.0±5.51% of control). Both treatments had no significant effect on cell death in control conditions.

5.2.1.3 ENT1 blockade increases extracellular adenosine levels and decreases cell death in retinal cell cultures under HG conditions

To assess a possible contribution of extracellular adenosine levels to cell death or survival we used NBMPR, a small molecule inhibitor of equilibrative nucleoside transporter (ENT) proteins that has been used to differentiate ENTs on the basis of their sensitivity to NBMPR action. ENT1 uptake of adenosine is blocked at NBMPR concentrations of 0.1 μM and below, while ENT2 is only affected by concentrations of NBMPR of 1 μM or higher (Tanaka et al., 2011). Because ENT1 was the transporter with increased expression levels in retinal cells under HG conditions, we used 0.1 μM a low concentration that only blocks ENT1. We have shown in 3.2.5 that the extracellular levels of adenosine are higher in retinal cells cultured in HG conditions as compared to control. This observation was again confirmed when, as shown in Figure 28, exposure of retinal cells to HG induced an increase in the extracellular levels of adenosine to 124.4±5.10% of control ($p<0.05$). A similar increase in the levels of extracellular adenosine was observed when retinal cells, cultured in control conditions, were exposed to NBMPR (137.8±10.19% of control, $p<0.01$). When the ENT1 inhibitor was applied to retinal cells cultured in HG conditions, the extracellular levels was further increased, attaining a maximum value of 153.4±4.62% of control ($p<0.001$). Therefore, in retinal cells exposed to the ENT1 inhibitor, both control and HG conditions experienced an increase in the extracellular levels of adenosine, although the increase

observed in HG conditions, when compared to control conditions, was only about 11% , contrasting with the 24% increase observed in HG vs. control without NBMRP.

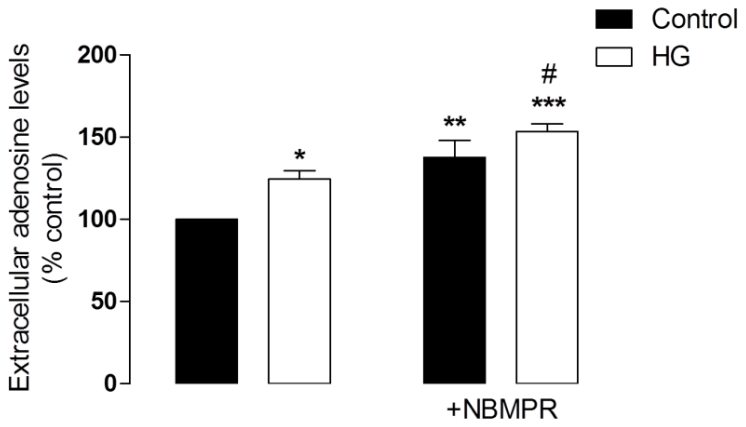

Figure 28: Effect of ENT1 blockade on extracellular adenosine levels in retinal cell cultures under HG conditions. NBMPR (0.1 μM) was added twice over a span of four days to block ENT1, before the levels of adenosine were measured in 20 μl of culture medium by HPLC and normalized for the protein content. Results are expressed as percentage of the control group. The mean±SEM of 3-5 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001; #p<0.05 in relation to HG.

The cell death observed in retinal cells cultured in HG was prevented by blockade of ENT1. As shown in Figure 29, after incubating cells with NBMPR, we observed a marked effect upon the elevated number of PI-positive cells normally seen under HG conditions, with the numbers decreasing to 103.5±6.34% of control. Inhibition of ENT1 had no significant effect on cell death observed in control conditions.

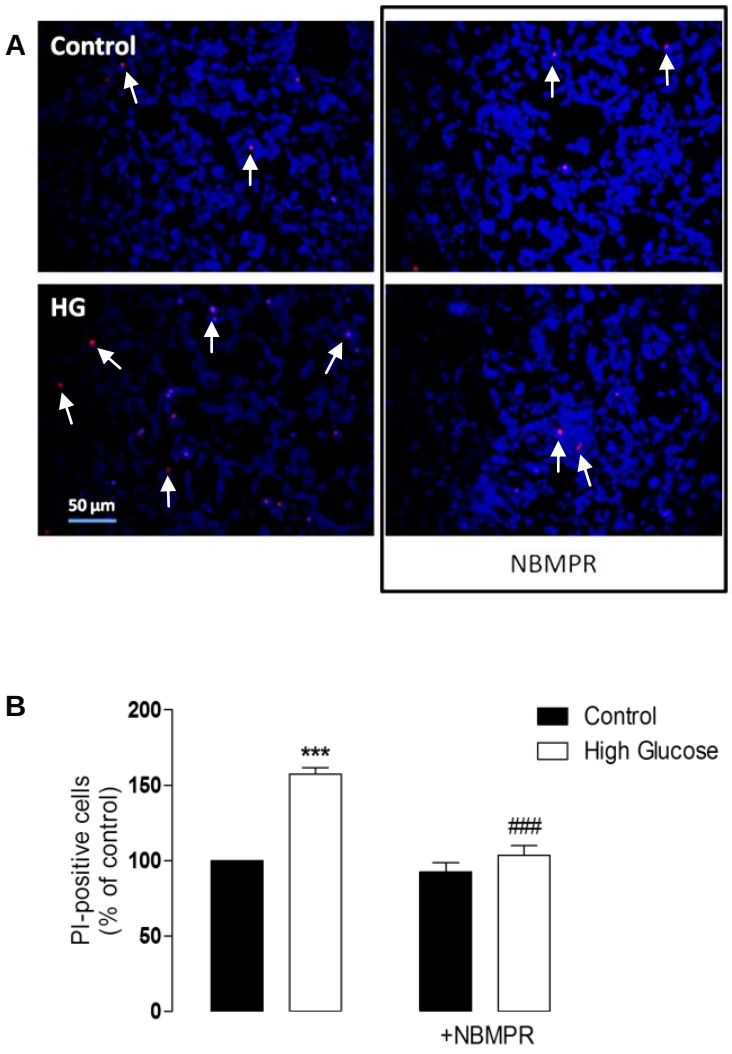

Figure 29: Effect of ENT1 blockade on cell death in retinal cell cultures under HG conditions. Cell death was evaluated in retinal cells after seven days of HG exposure by the PI method. NBMPR (0.1 µM) was added twice over a span of four days to block ENT1, before PI incubation. **(A)** Hoechst 33342-stained nuclei appear in blue, PI appears in red. Arrows denote dying cells, where PI and Hoechst 33342 co-localize at the nucleus. **(B)** Results are expressed as percentage of the control group. The mean±SEM of 3 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. ***p<0.001; ###p<0.001 in relation to HG.

5.2.2 *In vivo* inhibition of $A_{2A}R$ signaling

Following the protective effects of SCH 58261 in reducing cell death on retinal cells under HG conditions, we moved on to assess the effectiveness of $A_{2A}R$ inhibition in ameliorating the loss of retinal function and increased cell death characteristic of early

DR. We used rats diabetic for four weeks and aged matched controls, and administered a daily dose of SCH 58261 (i.p.) for the last seven days before performing ERGs, as described in Chapter 2. After the recordings, the animals were sacrificed, and the retinas extracted for qRT-PCR analysis and also for immunohistochemistry, to evaluate RGC loss and a possible influence of $A_{2A}R$ inhibition on the inflammatory environment of the diabetic retina. The mean animal weight and glycaemia at the time of ERG recordings are detailed in Table 4.

Table 4: Weight and blood glucose levels of animals at the end point of the experiment.

	Weight (g)	Blood glucose levels (mg/dL)
Control	355±26.5	103±3.5
Diabetic	281±29.5	483.2±24.8
Control + SCH 58261	354.8±18.1	108.5±7.5
Diabetic + SCH 58261	297±28.5	538.2±45.6

5.2.2.1 – $A_{2A}R$ blockade is able to reduce some of the alterations observed in scotopic ERG after four weeks of diabetes

With the animals kept in dark conditions previous to the procedure, the first ERG response measured was the scotopic ERG that assesses the function of the dark adapted retina. It is comprised of a series of spaced light flashes of increasing luminance against a dark background, from 0.0095 to 9.49 cd-s/m^2, and the resulting waveforms (Figure 30) were subsequently analyzed and filtered to yield the a-wave, the b-wave, and OPs. The negative a-wave is the first feature of the waveform and is assessed directly from it, without requiring post-treatment. It was clearly identifiable only for luminances starting at 0.03 cd-s/m^2. The a-wave recorded is generated mainly by rod photoreceptors and, as seen in Figure 31, it was not affected by diabetic conditions at four weeks of diabetes, with amplitude and latency values remaining similar to control in all degrees of luminance.

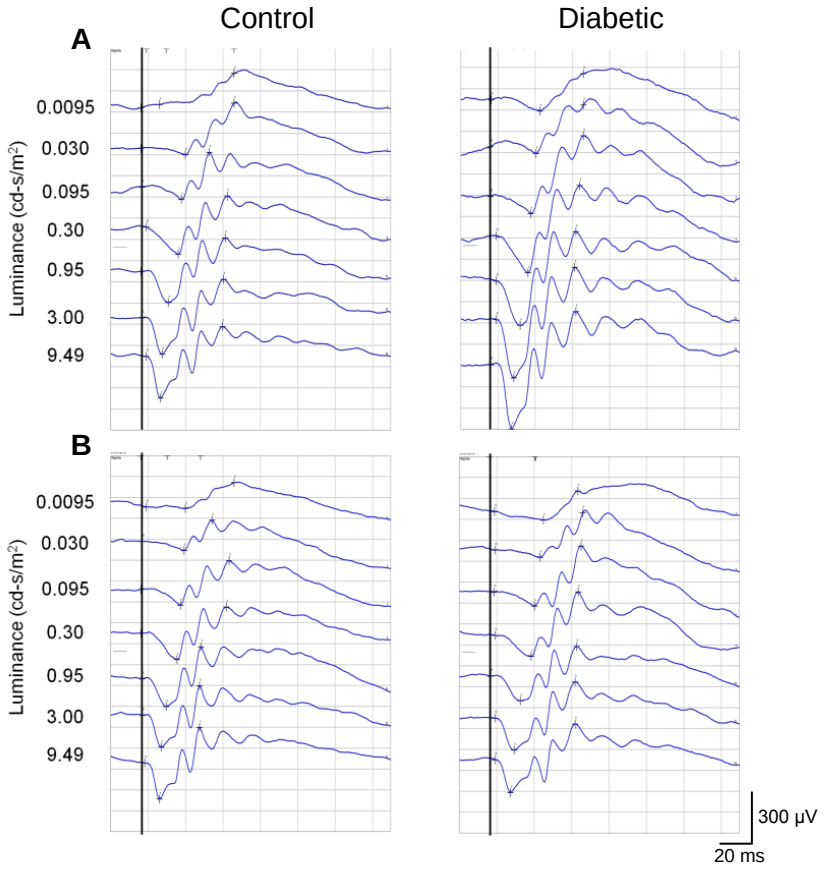

Figure 30: Example of individual scotopic ERG waveforms. ERG responses were induced by flash light stimuli of increasing luminance, from 0.0095 to 9.49 cd-s/m^2, four weeks after diabetes induction. The figure illustrates the raw waveforms without filters, for control and diabetic animals, with **(B)** or without **(A)** treatment with SCH 58261 (0.05 mg/kg), started one week before ERGs. Solid vertical lines indicate the onset of the light stimuli.

To evaluate the b-wave, we first applied to the waveform a high frequency cut off digital filter to remove the OPs. The scotopic b-wave is generated mainly by ON-bipolar cells, and diabetic animals show an increase in amplitude at all luminance points (Figure 32A and C), becoming statistically significant at 0.095 cd-s/m^2 (163.0±11.12% of control, $p<0.01$), while treatment with SCH 58261 retains amplitude levels closer to control, even at 0.095 cd-s/m^2 (118.3±17.22% of control). The same is visible in time to peak values (Figure 32B), where the diabetic b-wave shows an initial delay that becomes significant again at 0.095 cd-s/m^2 (121.8±9.06% of control, $p<0.05$). Although treatment with SCH 58261 was not effective in the overall time to peak performance of the b-wave, it was still able to hold latency values closer to control at 0.095 cd-s/m^2

(110.1±1.40% of control). The intensity-response function of the scotopic b-wave was fitted to the Naka-Rushton equation (Chapter 2), which empirically describes the amplitude rise in function of stimulus luminance in the dark-adapted retina. The asymptotic maximum response amplitude (R_{max}) reflects a general view of the functioning retina, and provides a gross comparison between groups. As seen in Figure 32C, the R_{max} values of diabetic animals are higher than control or diabetic animals treated with SCH 58261.

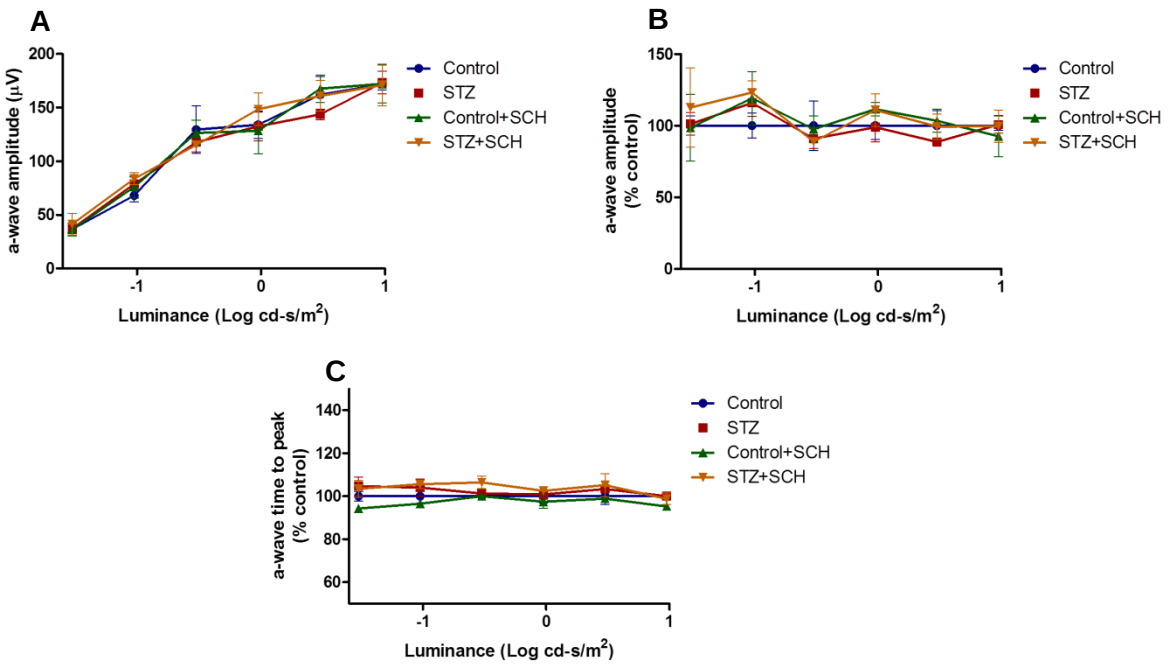

Figure 31: Effect of $A_{2A}R$ blockade on a-wave amplitude and time to peak of scotopic ERG. ERG responses were induced by six flash light stimuli of increasing luminance, from 0.03 to 9.49 cd-s/m^2, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). **(A)** Representation of the a-wave amplitude as a function of luminance. **(B)** Representation of the a-wave amplitude values in relation to the control group. **(C)** Time to peak values of the scotopic a-wave. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with two-way repeated measures ANOVA test and Bonferroni's multiple comparison test.

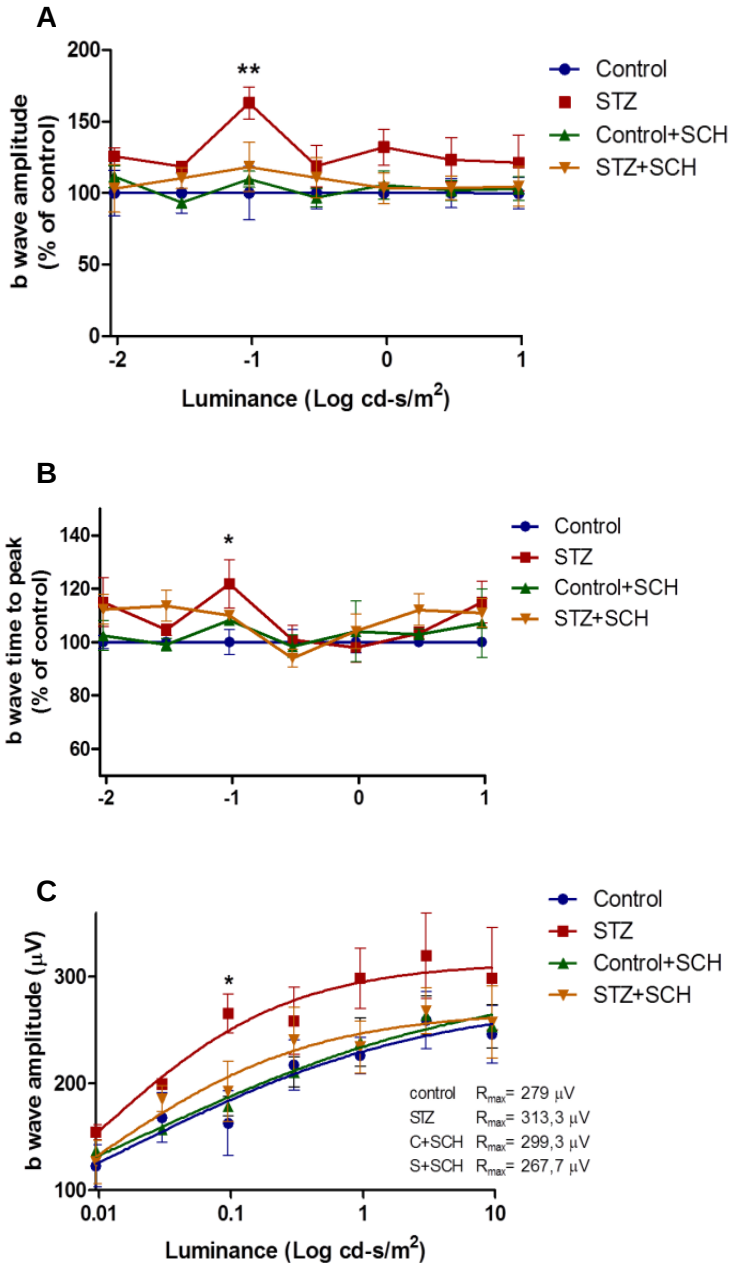

Figure 32: Effect of $A_{2A}R$ blockade on b-wave amplitude and time to peak of scotopic ERG. ERG responses were induced by 7 flash light stimuli of increasing luminance, from 0.0095 to 9.49 cd-s/m^2, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). **(A)** Representation of the b-wave amplitude values in relation to the control group. **(B)** Time to peak values of the scotopic b-wave. **(C)** The b-wave amplitude as a function of luminance. The lines represent the curve fitting of data points to the Naka-Rushton equation. Asymptotic maximum response (R_{max}) values are also presented. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with two-way repeated measures ANOVA test and Bonferroni's multiple comparison test. *p<0.05, **p<0.01.

After applying a low frequency cut-off digital filter to the original waveform, four OPs were isolated and analyzed. Although diabetic animals showed a general reduced amplitude in OP1 (Figure 33A), it was not statistically significant in any luminance points. OP2, OP3 and OP4 were not affected by either diabetes or the treatment, despite fluctuations of amplitude (Figure 33 B, C, D). The sum of all OP amplitudes (Figure 33E), shows that, overall, diabetic animals have decreased amplitudes from 0.300 cd-s/m^2 onwards, but the decrease is not statistically significant.

As for time to peak values, diabetic animals show increased latencies at all luminance points in all OPs, although not always with statistical significance. In OP2 (Figure 34B) the delay becomes significant for the two higher luminance points (107.4±2.33 and 108.3±2.35% of control, respectively, $p<0.05$), while the delay in OP1 (Figure 34A) and OP3 (Figure 34C) achieves significance at the highest luminance point of 9.49 cd-s/m^2 (109.2±2.74% of control, $p<0.01$, and 109.0±2.75% of control, $p<0.05$, respectively). The increased latency in OP4 was not significant in any luminance point (Figure 34D). Diabetic animals treated with SCH 58261 showed a tendency to recover time to peak values as luminance increased in OP2, and had non-significant, but consistent lower values than non-treated diabetic animals in OP3.

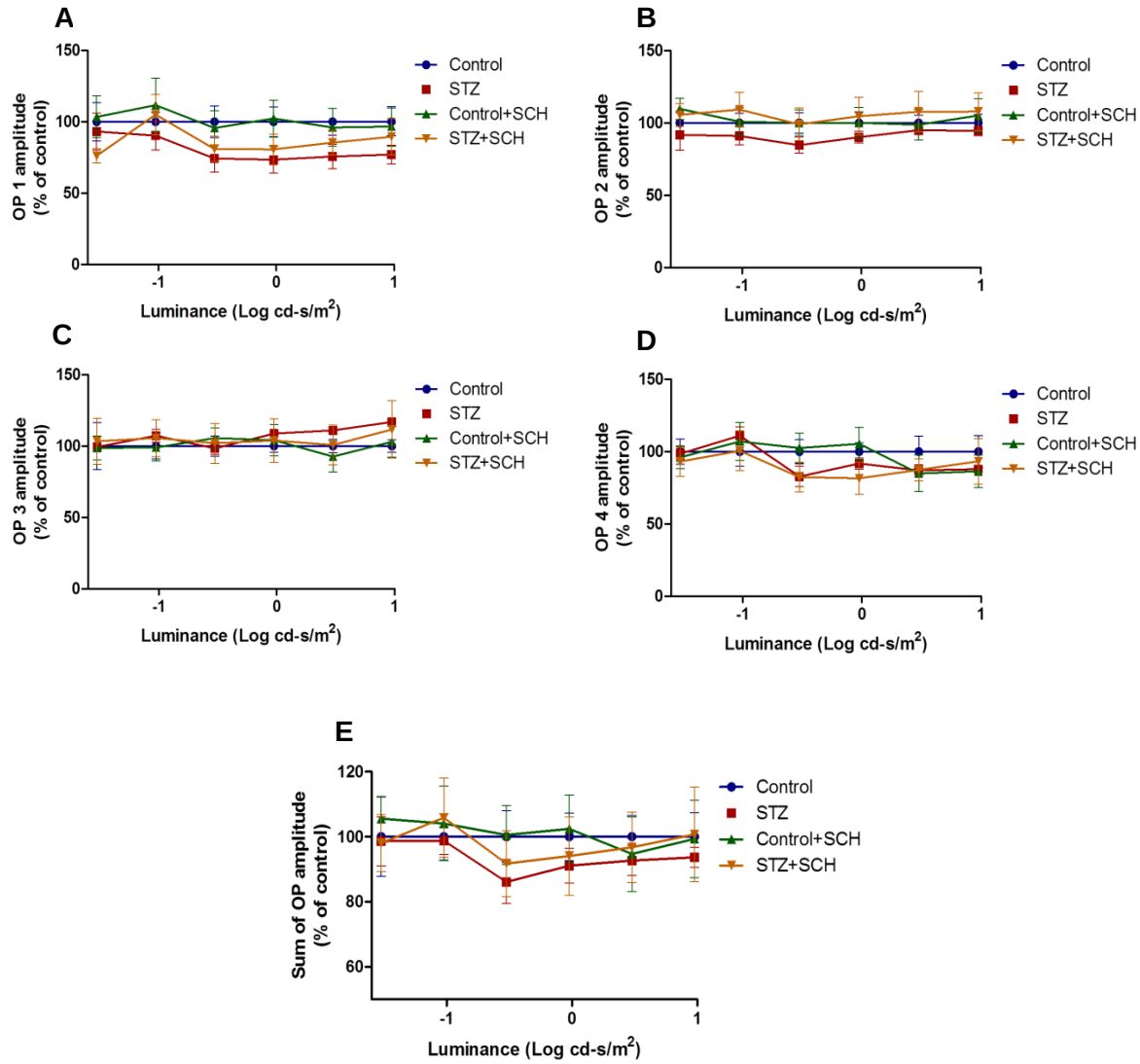

Figure 33: Effect of $A_{2A}R$ blockade on OP amplitude of scotopic ERG. ERG responses were induced by 6 flash light stimuli of increasing luminance, from 0.030 to 9.49 cd-s/m^2, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Four OPs were detected and the amplitude values assessed: OP1 **(A)**, OP2 **(B)**, OP3 **(C)**, and OP4 **(D)**. **(E)** Evaluation of the sum of OP amplitudes. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with two-way repeated measures ANOVA test and Bonferroni's multiple comparison test.

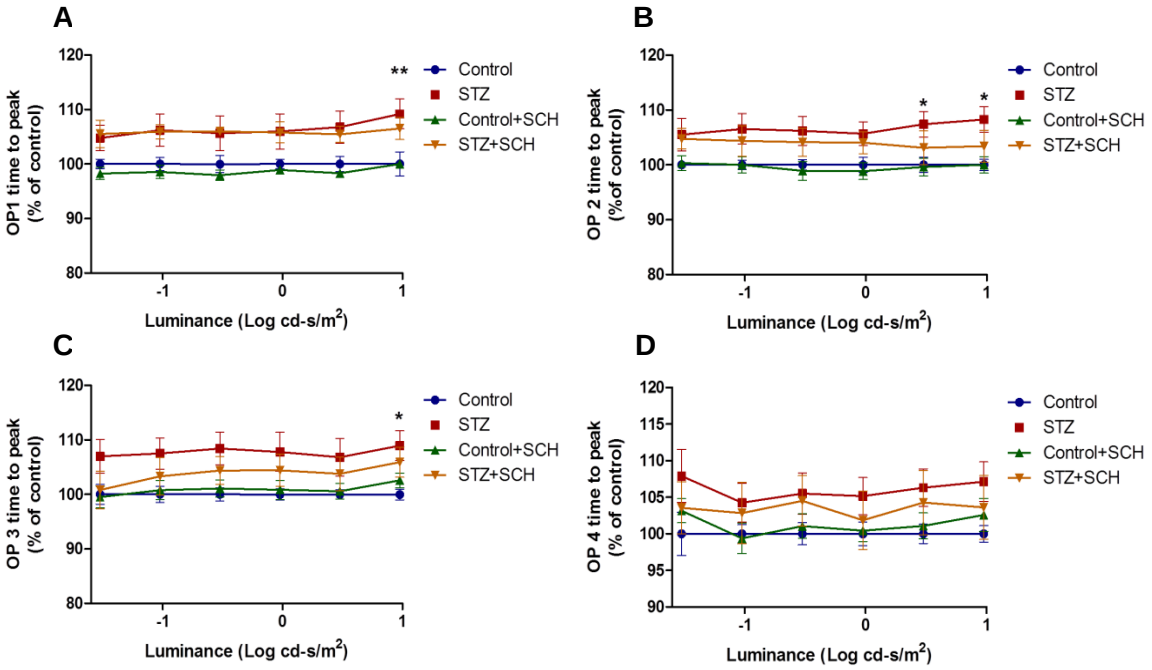

Figure 34: Effect of $A_{2A}R$ blockade on OP time to peak of scotopic ERG. ERG responses were induced by 6 flash light stimuli of increasing luminance, from 0.030 to 9.49 cd-s/m^2, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Four OPs were detected and the time to peak values assessed: OP1 **(A)**, OP2 **(B)**, OP3 **(C)**, and OP4 **(D)**. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with two-way repeated measures ANOVA test and Bonferroni's multiple comparison test. *p<0.05, **p<0.01.

5.2.2.2 The time to peak of b-wave during light adaptation is affected by four weeks of diabetes

After scotopic recordings, animals were light-adapted during 16 min, and the ERGs obtained during adaptation analyzed. Due to the low number of cone photoreceptors in rat retina, the a-wave was not identifiable in this procedure (Figure 35).

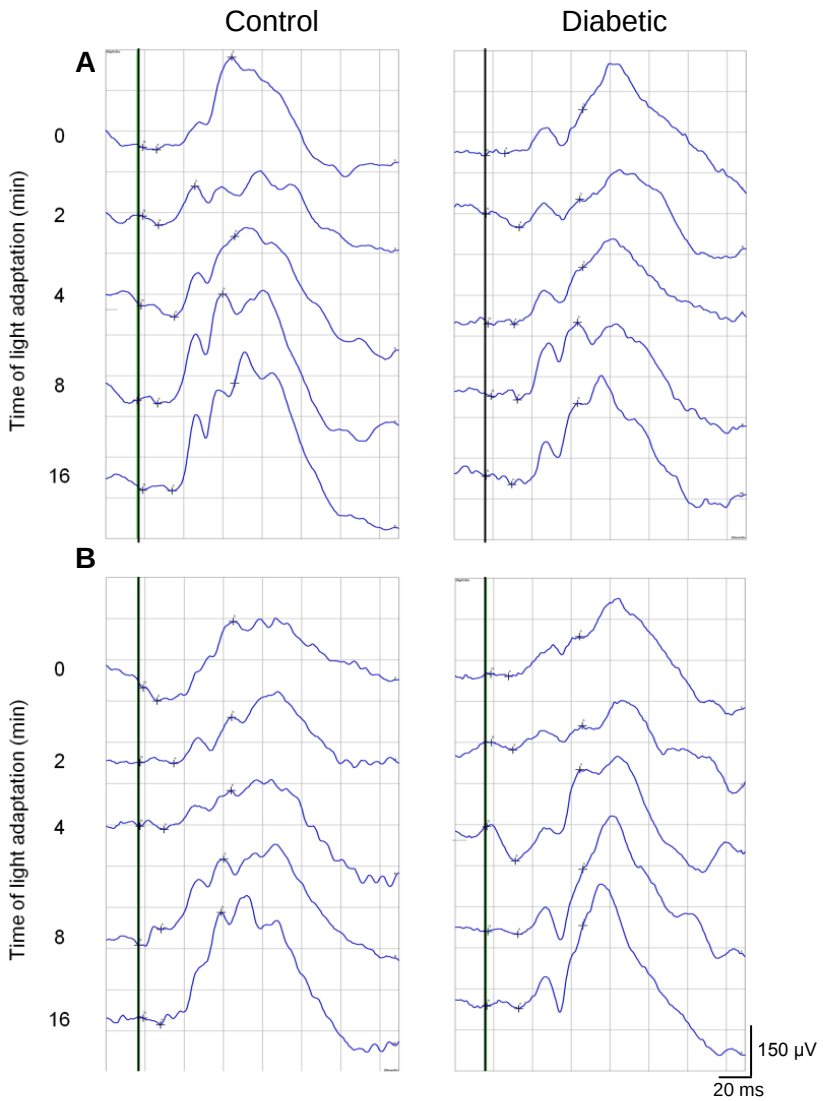

Figure 35: Example of individual ERG waveforms recorded during light adaptation. ERG responses were induced by a 9.49 cd-s/m^2 light stimulus at the onset (0 min) and at 2, 4, 8, and 16 min of light adaptation, four weeks after diabetes induction. The figure illustrates the raw waveforms without filters, for control and diabetic animals, with **(B)** or without **(A)** treatment with SCH 58261 (0.05 mg/kg), started one week before ERGs. Solid vertical lines indicate the onset of the light stimuli.

Regarding the b-wave amplitude (Figure 36A), there were no consistent differences between groups over time of adaptation, although diabetic animals started adaptation with higher values than control, consistent with the results obtained during scotopic recordings. As for time to peak values of the b-wave during light adaptation (Figure 36B), and consistent with the scotopic recordings, diabetic animals showed a significantly delayed time to peak response at the onset of light adaptation

(132.6±3.32% of control, p<0.01), a delay that was recovered as adaptation progressed. Control animals treated with SCH 58261 showed a tendency for decreased time to peak values on the second half of light adaptation.

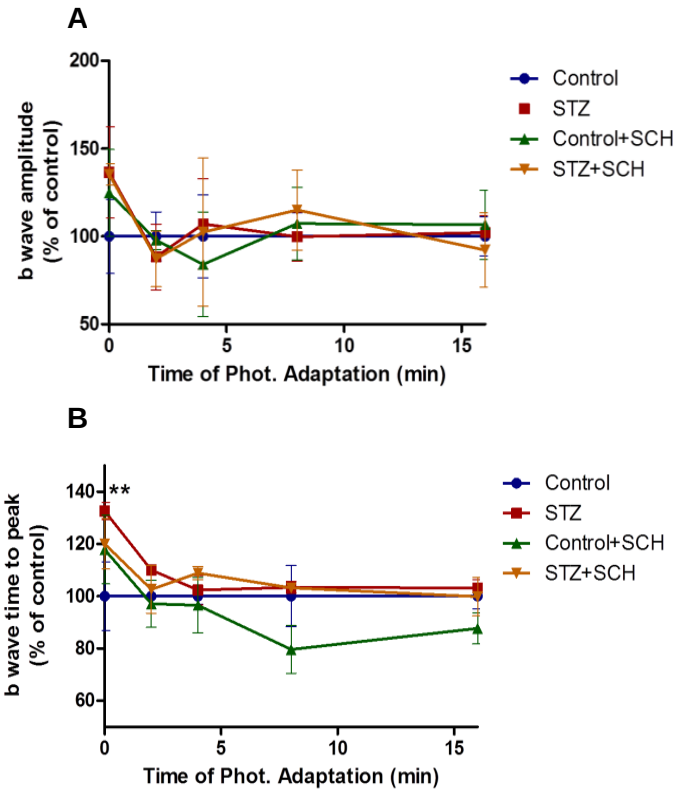

Figure 36: Effect of $A_{2A}R$ blockade on b-wave amplitude and time to peak of ERG during light adaptation. ERG responses were induced by a 9.49 cd-s/m^2 light stimulus at the onset (0 min) and at 2, 4, 8, and 16 min of light adaptation, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Amplitude **(A)** and time to peak **(B)** values were assessed. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with two-way repeated measures ANOVA test and Bonferroni's multiple comparison test. **p<0.01.

5.2.2.3 Four weeks of diabetes have a small impact on photopic ERG responses

After light adaptation, photopic ERGs were recorded and, as mentioned above, due to the low density of cone photoreceptors in rat retina, no a-wave was identifiable. Beyond that, only at higher light intensities, 3.00 and 9.49 cd-s/m^2, was the b-wave detected in

all animals and therefore only those two luminance points were evaluated (Figure 37). Regarding the b-wave amplitude at both luminance points (Figure 38 A, C), there was a tendency for increase that again was not significant, while diabetic animals treated with SCH 58261 retained amplitude values closer to control. As for time to peak values (Figure 38 B, D), there were no statistically significant differences between groups, despite a small delay of diabetic animals, both treated and untreated. As with photopic adaptation, control animals treated with SCH 58261 showed a tendency for decreased time to peak values at 9.49 cd-s/m^2.

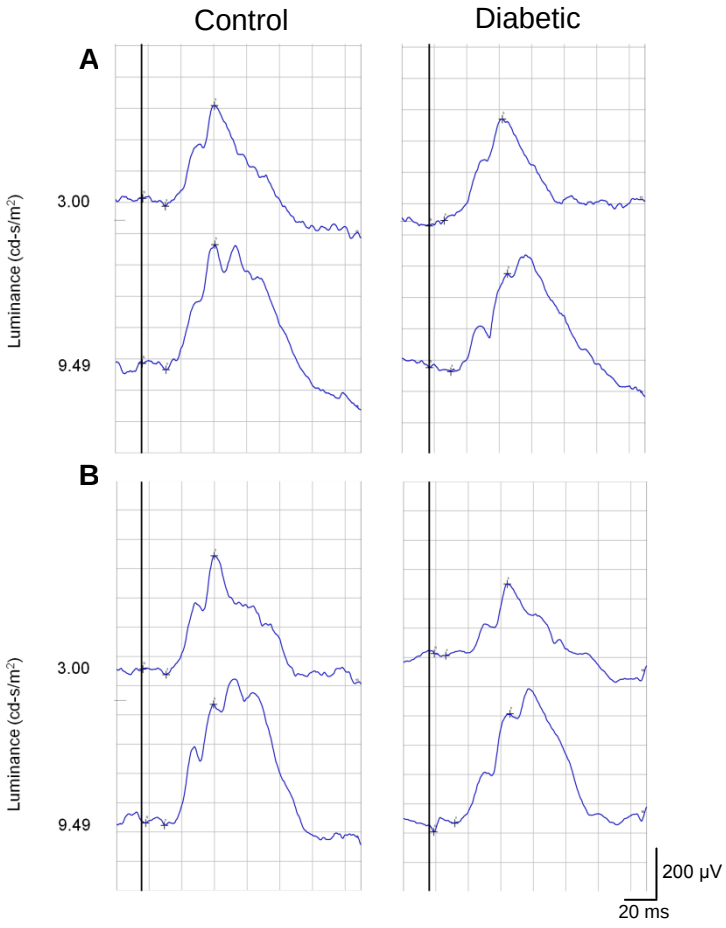

Figure 37: Example of individual photopic ERG waveforms. ERG responses were induced by bright light flashes with intensities of 3.00 and 9.49 cd-s/m^2, four weeks after diabetes induction. The figure illustrates the raw waveforms without filters, for control and diabetic animals, with **(B)** or without **(A)** treatment with SCH 58261 (0.05 mg/kg), started one week before ERGs. Solid vertical lines indicate the onset of the light stimuli.

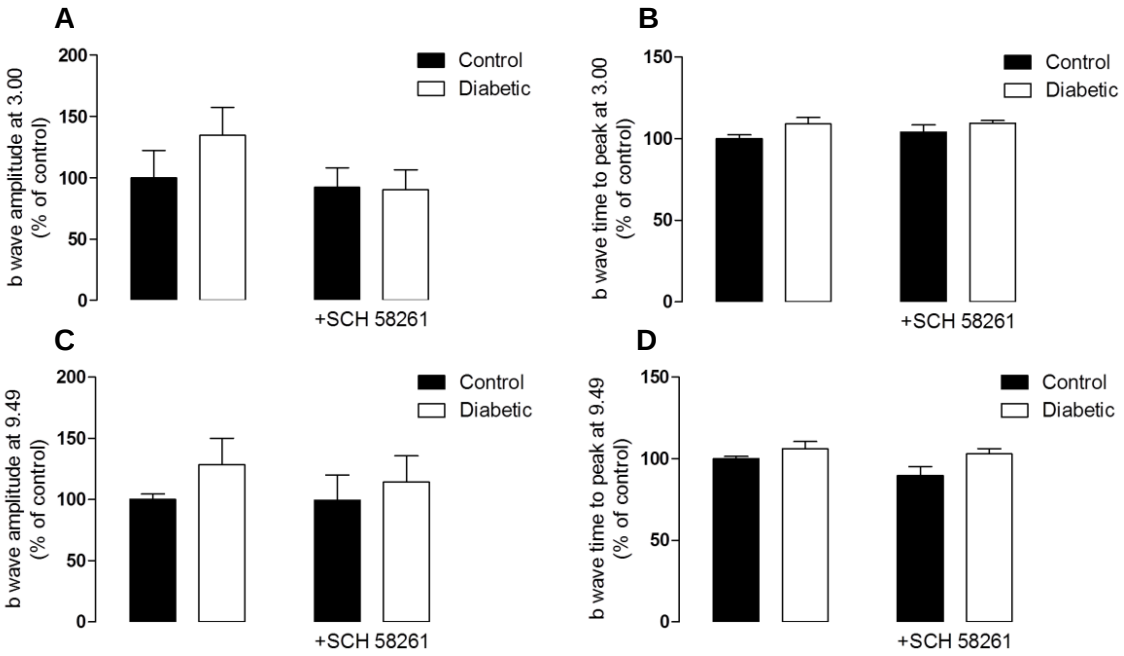

Figure 38: Effect of $A_{2A}R$ blockade on b-wave amplitude and time to peak of photopic ERG. ERG responses were induced by 3.00 and 9.49 cd-s/m^2 flash light stimulus, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Amplitude **(A, C)** and time to peak **(B, D)** values were assessed. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA test and Bonferroni's multiple comparison test.

The OP amplitudes registered in light adapted animals are much smaller than those obtained in scotopic conditions. Nevertheless, 3 OPs were identified in the photopic waveform at the highest luminance point of 9.49 cd-s/m^2 and were then evaluated (Figure 39). There were no statistically significant differences in amplitude values of OPs between groups. As for time to peak values (Figure 40), diabetic animals again showed a tendency for increased values at all OPs, although only in OP4 (Figure 40C) was the increase statistically significant (109.1±2.34% of control, $p<0.01$). In all 3 OPs recorded, diabetic animals treated with SCH 58261 showed no amelioration and had similar values to untreated animals.

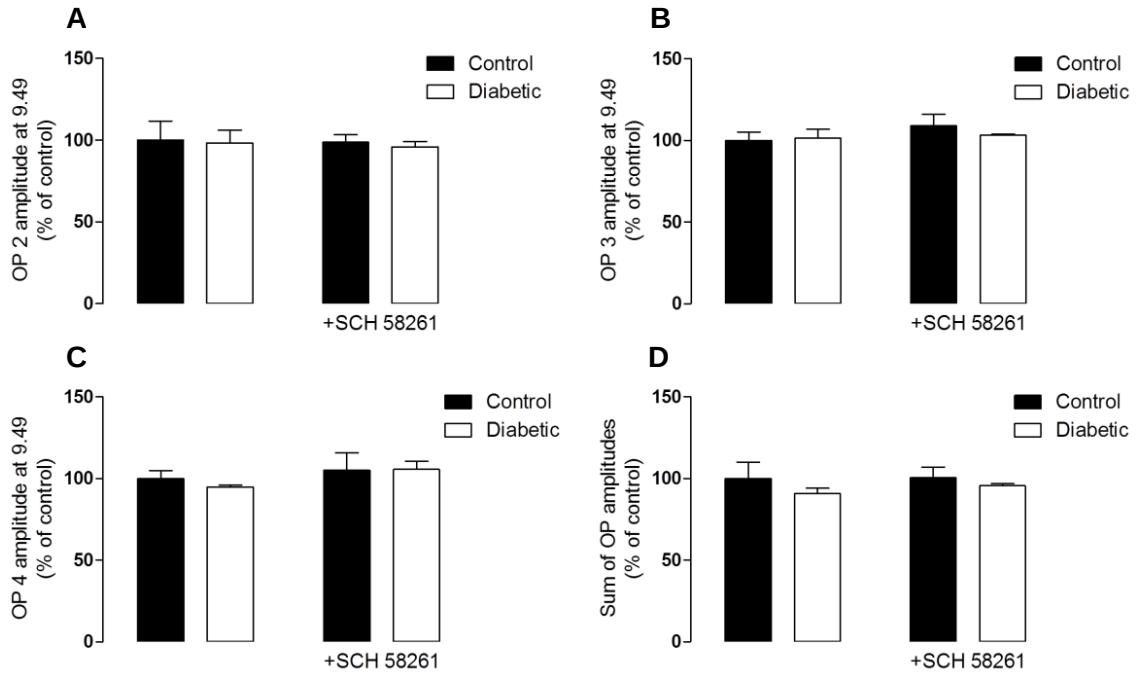

Figure 39: Effect of $A_{2A}R$ blockade on OP amplitude of photopic ERG. ERG responses were induced by a 9.49 cd-s/m^2 flash light stimulus, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Three OPs were detected and the amplitude values assessed: OP2 **(A)**, OP3 **(B)**, and OP4 **(C)**. **(D)** Evaluation of the sum of OP amplitudes. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA test and Bonferroni's multiple comparison test.

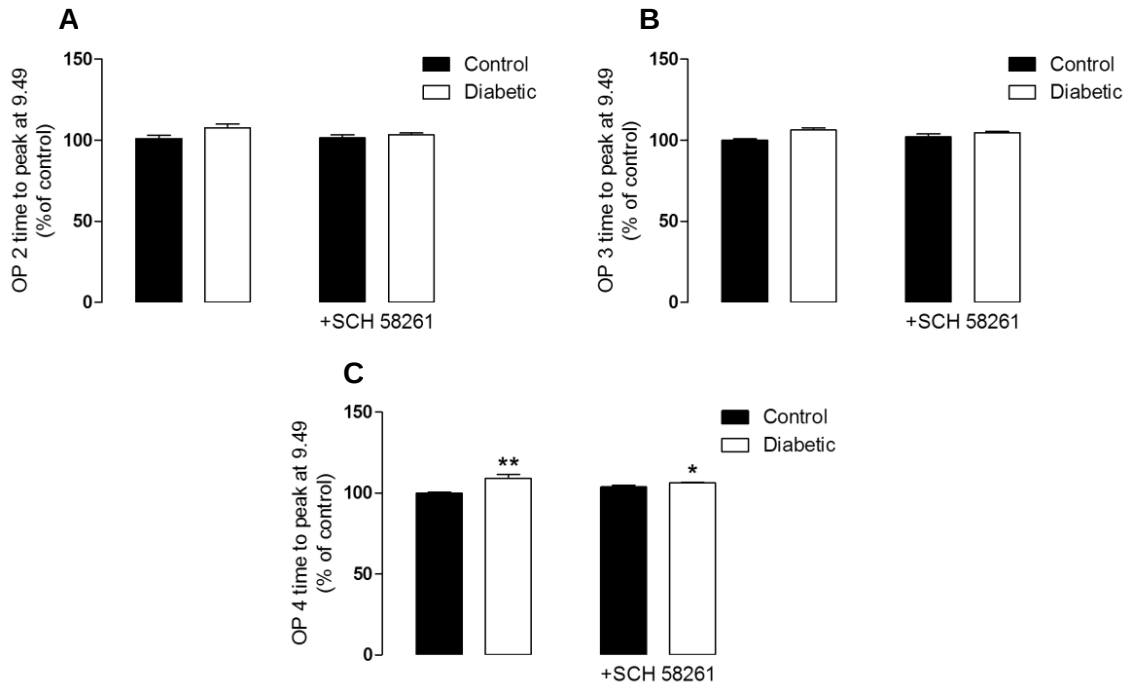

Figure 40: Effect of $A_{2A}R$ blockade on OP time to peak of photopic ERG. ERG responses were induced by a 9.49 cd-s/m^2 flash light stimulus, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Three OPs were detected and the time to peak values assessed: OP2 **(A)**, OP3 **(B)**, and OP4 **(C)**. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA test and Bonferroni's multiple comparison test. *p<0.05, **p<0.01.

5.2.2.4 Four weeks of diabetes do not affect the photopic flicker ERG responses

The final step of the ERG recording was the photopic flicker, with evaluates the cone-driven response to high temporal frequency light stimuli in photopic conditions. The maximum harmonic amplitude and phase values at 3.00 cd-s/m^2 and 9.49 cd-s/m^2 were determined from the original waveforms (Figure 41) induced by bright light flashes administered at a temporal frequency of 6.3 Hz. A seen in Figure 42, there were no significant alterations to the maximum amplitude or phase values between groups, despite a tendency for increased amplitude of untreated diabetic animals at 3.00 cd-s/m^2 (111.0±8.31% of control) (Figure 42A).

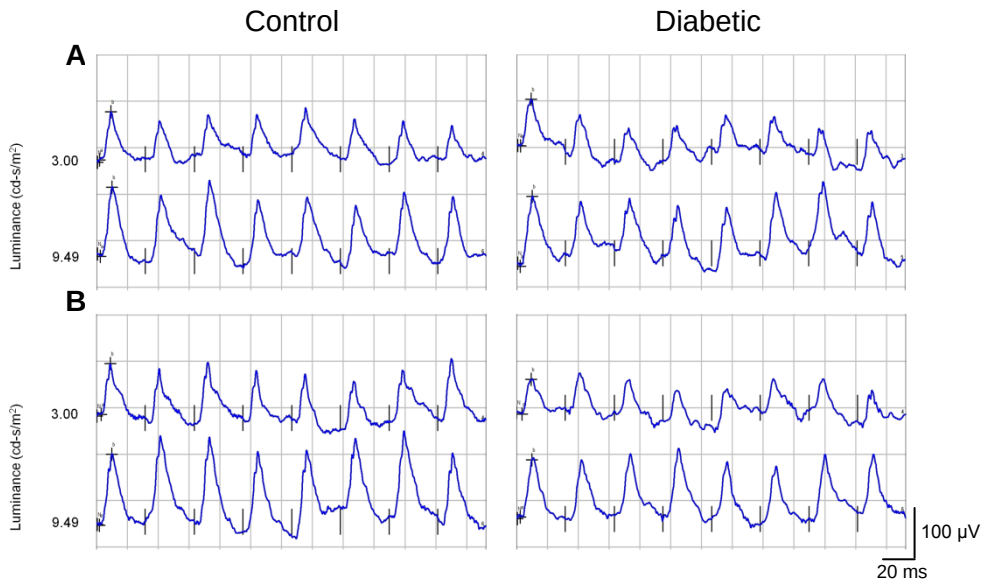

Figure 41: Example of individual photopic flicker recordings. Flicker responses were induced by bright light flashes with intensities of 3.00 and 9.49 cd-s/m^2 administered at 6.3 Hz, four weeks after diabetes induction. The figure illustrates the raw waveforms without filters, for control and diabetic animals, with **(B)** or without **(A)** treatment with SCH 58261 (0.05 mg/kg), started one week before ERGs. Small solid vertical lines indicate the flash light stimuli.

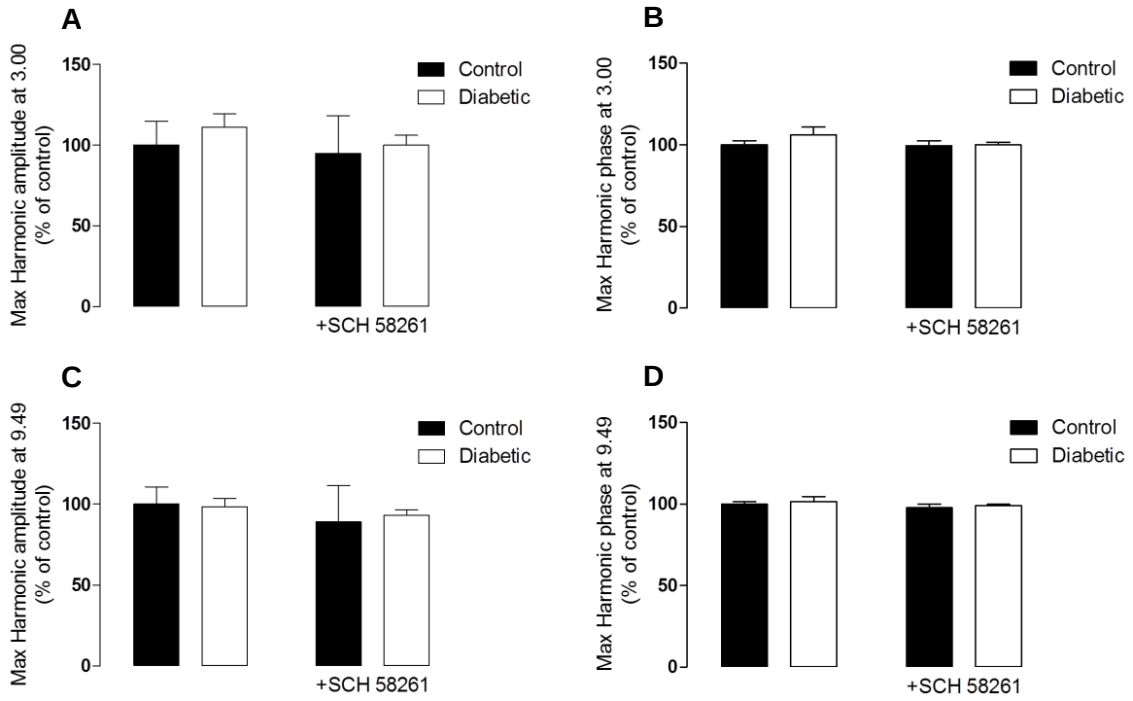

Figure 42: Effect of $A_{2A}R$ blockade on harmonic amplitude and phase of photopic flicker ERG. Flicker responses were induced by bright light flashes with intensities of 3.00 and 9.49 cd-s/m^2 administered at 6.3 Hz, four weeks after diabetes induction, and after one week of SCH 58261 treatment (0.05 mg/kg). Maximum harmonic amplitude **(A, C)** and phase **(B, D)** values were assessed for each intensity. Both eyes were recorded and used as replicas. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA test and Bonferroni's multiple comparison test.

5.2.2.5 $A_{2A}R$ blockade is able to increase RGC cell survival after four weeks of diabetes

It is well established that loss of retinal neurons is already extensive by the time the first diagnostic signs of DR appear (Simó et al., 2014). Of the retinal cell populations, RGCs are considered the most sensitive to disruptions in homeostasis, and the loss of RGCs is a characteristic of the early stages of DR (Kern and Barber, 2008). Therefore, an evaluation of the RGC population is not only an efficient method to assess the health of the neuroretina in the early stages of DR, but also to evaluate the effect that the treatment with the $A_{2A}R$ antagonist has on neuronal cell death in early DR. For this, we used retinal slices obtained from the same animals that were

treated with SCH 58261 for seven days, and performed immunohistochemistry to stain slices for Brn3a, a selective marker that allows the isolation of RGC population (Nadal-Nicolás et al., 2009).

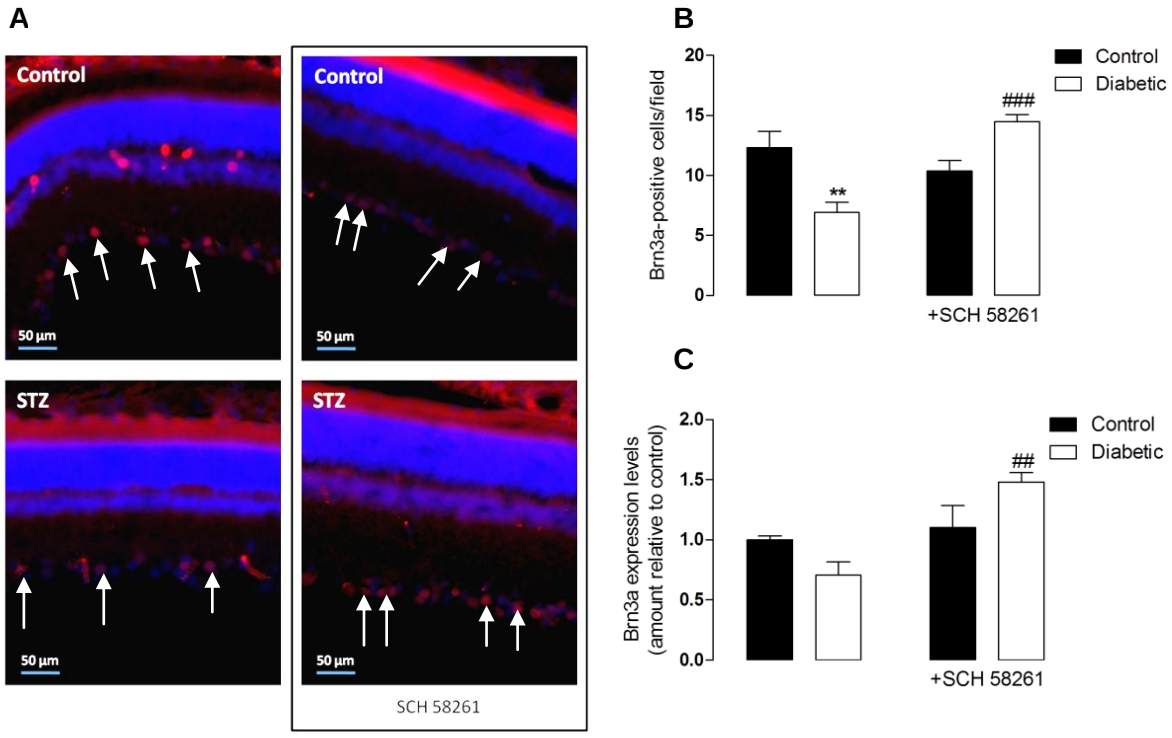

Figure 43: Effect of $A_{2A}R$ blockade on RGC survival. (A) Retinal slices were stained for the RGC marker Brn3a, four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg), to evaluate RGC survival. DAPI-stained nuclei appear in blue, Brn3a appears in red. Arrows denote RGCs, where Brn3a and DAPI co-localize at the nucleus. **(B)** Calculation of the number of RGCs per condition, represented as cells per field. **(C)** The expression levels of Brn3a in the retina were assessed by qRT-PCR four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test. **p<0.01; ##p<0.01, ### p<0.001 in relation to non-treated diabetic animals.

As seen in Figure 43A, Brn3a stained cells were present in the GCL, and diabetic animals showed a significant loss of Brn3a-positive cells, making it evident that loss of RGCs is already underway after four weeks of diabetes (from 12.3±1.37 ganglion cells/field in control, to 6.92±0.84 ganglion cells/field in diabetic retinas, p<0.01, Figure 43B). Diabetic animals treated with SCH 58261, on the other hand, showed no loss of

Brn3a-positive cells (14.49±0.58 ganglion cells/field), underlining the preventive effect of $A_{2A}R$ blockade on RGC loss. This result was corroborated by q-RT-PCR analysis of Brn3a, where results bore a resemblance (Figure 43C): there was a loss of Brn3a expression in diabetic retinas that, despite levels decreasing to 0.70±0.11 in relation to control, was short of significance, while in treated diabetic animals the expression levels of Brn3a increased to 1.48±0.08 in relation to control ($p<0.01$ in relation to Diabetic).

5.2.2.6 $A_{2A}R$ blockade has no effect on TNF and IL-1β expression levels after four weeks of diabetes

$A_{2A}R$ is reported to modulate several inflammatory processes, from control of proliferation to production and release of inflammatory cytokines (Haskó and Cronstein, 2004). Therefore, we assessed the expression levels of two pro-inflammatory cytokines, TNF and IL-1β, which are increased in diabetic conditions in the retina (Aveleira et al., 2010; Aveleira et al., 2010), and can be modulated by $A_{2A}R$ activation (Haskó et al., 2008). In the expression of both TNF and IL-1β in diabetic retinas, as seen in Figure 44, we observed a tendency for increased expression, although a high error prevented statistical significance (1.51±0.57 fold of control for TNF and 1.75±0.28 fold of control for IL-1β). Treatment of diabetic animals with SCH 58261 was not enough to revert the expression levels on both cases, with TNF and IL-1β levels seemingly unaffected by the treatment.

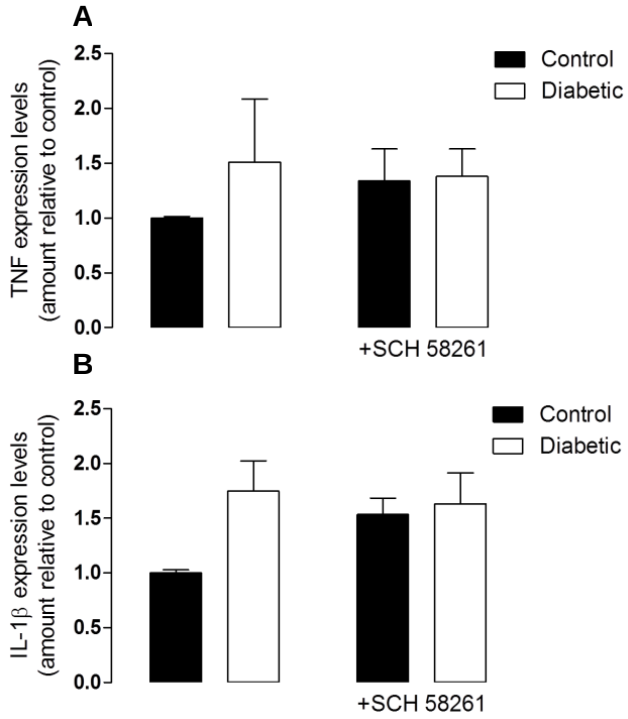

Figure 44: Effect of $A_{2A}R$ blockade on TNF and IL-1β expression levels. The expression levels of TNF **(A)** and IL-1β **(B)** in the retina were assessed by qRT-PCR four weeks after diabetes induction and after one week of SCH 58261 treatment (0.05 mg/kg). Experiments were carried out in triplicate. The mean±SEM of 3-4 independent experiments was analyzed with one-way ANOVA and Tukey's multiple comparison test.

5.3 Discussion

5.3.1 *In vitro* modulation of A_1R and $A_{2A}R$ signaling, and ENT1 blockade

A_1R and $A_{2A}R$ are frequently involved in the same processes, more often by opposite and sequential regulatory actions, creating a signaling system that is self-modulated (Ghiardi et al., 1999; Rogachev et al., 2006). The protective effects of the activation of A_1R and inhibition of $A_{2A}R$ on retinal cells under HG conditions that we observed seem to point to a similar process. A_1R activation can exert protective effects most likely through the inhibition of excitatory neurotransmission (MacGregor et al., 1998; Li and Henry, 2000). It is a well known fact that diabetic conditions in the retina affect the glutamatergic signaling system, from increased glutamate levels (Ambati et

al., 1997; Kowluru et al., 2001), altered expression of glutamate receptor subunits (Santiago et al., 2008, 2009), to impaired glutamate metabolism (Gowda et al., 2011) and increased glutamate release (Santiago et al., 2006a), and these alterations have been linked to the retinal degeneration in the diabetic retina (Lieth et al., 1998). A_1R has a well documented role in controlling and inhibiting the excessive release of glutamate (MacGregor et al., 1998), and its activation is often beneficial in neurodegenerative situations because of this (Mitchell et al., 1995; Nakav et al., 2008), putting forth the hypothesis that the inhibition of excessive glutamate release in HG conditions may be the main drive behind the protective effects of A_1R observed in our work. However, we measured, by HPLC, the extracellular levels of glutamate in retinal cell cultures under HG conditions, with or without A_1R modulation, and did not observe significant alterations (data not shown). It is possible that the method was not the most appropriate for the type of cell culture, and an optimization of the procedure is needed before repeating the experiment. Another protective effect of A_1R activation can be the inhibition of Müller cell swelling that occurs in HG conditions (Uckermann et al., 2006). The fact that an inhibition of A_1R signaling had no effect on cell death under HG conditions means that, if the increased expression of A_1R observed in those conditions was part of a protective mechanism, it was either restricted to some cell populations, and therefore diluted in a mixed primary cell culture, or was largely ineffective, and greater levels of activation were required for an evident protection to occur.

$A_{2A}R$ inhibition was shown to be similarly effective in preventing the increase in cell death observed in HG conditions. This effect may be through an inhibition of excitatory transmitter release, the same process that confers $A_{2A}R$ inhibition its protective effects in ischemia/reperfusion events (Ghiardi et al., 1999), and other conditions such as Parkinson's and Huntington's diseases (Popoli et al., 2002). $A_{2A}R$ has also been shown to decrease glutamate uptake, in another mechanism that allows this receptor to influence and increase the accumulation of extracellular glutamate (Matos et al., 2013).

Another possible mechanism may be the influence that $A_{2A}R$ signaling can have on activated microglia: Several studies have shown that although $A_{2A}R$ actions on resting microglia are generally subdued (Saura et al., 2005; Santiago et al., 2014), they becomes relevant upon activation, inducing increased NO release (Saura et al., 2005) and NO synthase activity (Dai et al., 2010), as well as controlling process retraction and the speed reaction to an insult (Orr et al., 2009; Santiago et al., 2014).

The blockade of ENT1 with NBMPR was also shown to have protective effects, highlighting the hypothesis that the rise in adenosine levels present in cultures under HG conditions may be part of a protective feedback mechanism against the loss of homeostasis. It is known that adenosine levels rise quickly in response to insults, however, adenosine signaling is programmed to work as a transient response, and chronic elevated levels of adenosine not only disrupt signaling mechanisms, but also become a source of toxicity (Ghiardi et al., 1999; Blackburn, 2003). Therefore the increase of nucleoside transporter levels after an insult can be a mechanism to lower adenosine levels and recover homeostasis (Cui et al., 2013), and it is possible that this is the process behind the increased expression levels of ENT1 in retinal cell cultures under HG conditions. But this mechanism is not always protective: It was shown that repeated exposure to hypoxia causes an increased expression of ENT1 and a decrease in adenosine levels, and that this annuls the neuroprotective effects of hypoxic preconditioning (Cui et al., 2013). In this study, it was determined that the neuroprotective effects of increased adenosine levels that result from preconditioning were achieved through A_1R activation, and it is possible that the same is happening here: the decrease in cell death provided by NBMPR in HG conditions may be caused by the increased extracellular adenosine levels and the activation of A_1R, since it was demonstrated that A_1R activation can also reduce cell death in HG conditions, while $A_{2A}R$ activation did not protect, but also did not exacerbate the damage. Because of the potential toxicity of chronic elevated levels of adenosine, this method of ENT1 inhibition

by itself would not be the best choice in the long run, and direct action on receptors would be a preferable approach.

Both A_1R activation and $A_{2A}R$ inhibition proved to be successful in decreasing cell death in retinal cultures under HG conditions, however, because of the severe increase in $A_{2A}R$ receptor levels observed in retinal cell cultures under HG conditions and in diabetic retinas after one week of diabetes, a blockade of this receptor becomes a promising target to study *in vivo* and determine if it can also confer not only protection against cell death in diabetic retinas, but also improve overall retinal function.

5.3.2 *In vivo* inhibition of $A_{2A}R$ signaling

As mentioned previously, although some studies have detected alterations to the ERG recordings at early time points of diabetes, most alterations are only detected after eight or even ten weeks of diabetes. In our time point of four weeks we have determined that diabetes affects the ERG recordings, and that the function of the dark adapted retina is the most affected by diabetes, as evidenced by the fact that most alterations observed were in scotopic ERG recordings, while photopic ERGs were mostly unaffected, although this may also be influenced by the low number of animals used for this study, since some alterations were visible, but without statistical significance. The dark adapted retina is more metabolically active than in the light, mostly due to rod photoreceptor activity, which results in greater oxygen and energy consumption, and neurotransmitter release (Arden et al., 2005), and it has been suggested that DR is exacerbated during dark adaptation because of the high activity of photoreceptors which can decrease oxygen levels, leading to an increase in VEGF and its angiogenic effects on retinal vasculature. Beyond that, photoreceptors can generate, or regulate the levels of reactive oxygen species, as well as the synthesis of inflammatory proteins in diabetes (Du et al., 2013), contributing to the overall inflammatory and neurotoxic environment characteristic of DR. A study made in mice

lacking rhodopsin (which causes photoreceptor degeneration) revealed that DR development in these animals was stunted (de Gooyer et al., 2006), and diabetic patients with retinitis pigmentosa (as a result of photoreceptor degeneration) also had less severe DR than other diabetic patients (Arden et al., 2005). In addition, low light intensities during sleep are increasingly being recognized as a potential therapeutic tool against DR (Arden et al., 2010, 2011; Tang et al., 2013).

Of the alterations registered the most significant, and perhaps most unexpected one, was the increase in b-wave amplitude in scotopic responses, because when b-wave amplitude is shown to be altered in diabetic rodent retinas, most recordings show a decrease in scotopic b-wave amplitude (Hancock and Kraft, 2004; Pardue et al., 2014). However, the time points used are commonly above eight weeks of diabetes, so it is possible that four weeks of diabetes may not be enough to elicit that decrease. As for the increase observed, there was no concomitant increase in a-wave amplitude, which can rule out a-wave amplitude as an influence on the rise in b-wave amplitude, suggesting that a negatively-contributing component of the b-wave is affected. This may be related to impaired function of third-order neurons: it has been shown that disruption of signaling mechanisms from ganglion and amacrine cells can increase the b-wave amplitude (Dong and Hare, 2000). In fact, negative feedback mechanisms regulated by $GABA_A$ and glycine receptors control the normal b-wave amplitude (Kapousta-Bruneau, 2000; Frankfort et al., 2013), and loss of RGCs and amacrine cells can cause this control to loosen (Frankfort et al., 2013), resulting in increased b-wave amplitude, although impairment to this process can occur without overt amacrine cell loss (Dijk et al., 2004; Gunn et al., 2011). To further strengthen this hypothesis, a study in rats after eight weeks of diabetes revealed a significant decrease in the release of GABA in retinal synaptossomes (Baptista et al., 2011). It is possible that, at four weeks of diabetes, there is already impairment of amacrine cells and RGCs function, causing a transient increase in b-wave amplitude, before the progression of the disease causes the decrease in b-wave amplitude, masking the effects of these feedbacks' impairment.

While amacrine cell function, in a general way, can be reflected in OPs amplitude and time to peak, a possible method to directly assess RGC function is the recording of the positive scotopic threshold response. The scotopic threshold response is a small postreceptoral potential of opposite polarity to the b-wave that appears in very weak light flashes, and is affected directly by RGCs (Bui and Fortune, 2004). However, the equipment that was available to us was unable to register this parameter, and thus we had no means of directly assessing the impact of RGCs on retinal function.

The recovery provided by SCH 58261 treatment on diabetic animals may have been tentative overall, but seemed to show potential, especially in the case of the scotopic b-wave amplitude increase, putting forth the notion that $A_{2A}R$ blockade may be having a significant effect on third order neurons. In fact, when retinal slices were stained for the RGC marker Brn3a, and expression levels of the same marker were assessed, it was revealed that $A_{2A}R$ blockade prevented the loss of RGCs caused by diabetic condition. Although the values may have been inflated due to the low number of animals used for the experiment, it is still evident that the inhibition of $A_{2A}R$ signaling is beneficial for the survival of RGCs in diabetic conditions. It would be interesting to evaluate amacrine cell survival using a similar method, since the tendency for decreased amplitude of scotopic OPs and the significant delays in OP time to peak, along with the increased b-wave amplitude, are signs of impaired amacrine cell function. The $A_{2A}R$ blockade was able to moderately reduce time to peak delays in higher light intensities, particularly in OP2 and OP3, suggesting a possible influence on amacrine cell survival and function. It is possible that an increase in the number of animals used for the experiment would ameliorate the inherent error to working *in vivo* and cause the effects of SCH 58261 treatment to become more explicit.

There are several hypotheses to explain the effects of $A_{2A}R$ blockade on retinal function and RGC survival. $A_{2A}R$ inhibition could be interfering with inflammatory processes (Santiago et al., 2014), and reducing the pro-inflammatory environment characteristic of DR. To assess this, we evaluated the expression levels of two pro-

inflammatory cytokines, TNF and IL-1β that are up-regulated in DR and are involved in the increased cell death prevalent in diabetic retinas (Aveleira et al., 2010; Costa et al., 2012). Despite the evident rise observed in expression of both cytokines in diabetic retinas, due to the small number of animals used in this study, statistical significance was not attained. As for the treatment with SCH 58261, the expression levels of both TNF and IL-1β were not affected by $A_{2A}R$ blockade in diabetic retinas. Although an increase in the number of animals would be needed to confirm these results, it is possible that a direct action on inflammatory processes may not be the main method behind $A_{2A}R$ blockade actions in diabetic animals, for even other mechanisms regulating activated microglia that $A_{2A}R$ blockade could be interfering with would culminate in a decrease of pro-inflammatory cytokine production, of which TNF and IL-1β levels are normally used as markers. However, it would be useful to assess the levels of the pro-inflammatory transcription factor NF-κB which, beyond inducing the expression of many cytokines, chemokines and other pro-inflammatory components, it can also regulate the levels of adenosine receptors and be regulated by their signaling. Conversely to the results in diabetic retinas, $A_{2A}R$ blockade in control retinas showed a tendency to increase TNF and IL-1β expression levels, and this is perhaps in accordance with the view that $A_{2A}R$ signaling has different outcomes depending on the activation state of microglia (Santiago et al., 2014). This tendency for increase in TNF and IL-1β expression in control retinas may indicate that $A_{2A}R$ blockade is pro-inflammatory in resting microglia.

$A_{2A}R$ inhibition effects could also be due to its actions on neurotransmitter release and glutamate uptake (Matos et al., 2013). Not only a decrease of the excitotoxic environment could help the survival of RGC, among other cell types, but also could decrease glutamate release at the photoreceptor level, reducing the activity in dark adapted retina (Arden et al., 2005). Since no alterations were observed in the scotopic a-wave between groups, however, this last hypothesis can be discarded, and it is most

likely that any neuroprotective benefits of $A_{2A}R$ blockade are being felt in the inner retina.

It is also possible at the time point of four weeks, that $A_{2A}R$ blockade could already be having a protective effect through the inhibition of VEGF release. VEGF is produced largely by glia (mainly Müller cells) and neurons, and forms a link between neuroglia and vasculature (Ferrara et al., 2003). Its levels are elevated in DR and it is implicated in the increase of permeability and neovascularization in advance stages of DR (Jardeleza and Miller, 2009; Schlingemann and Witmer, 2009). However, VEGF has the potential to be damaging even in early stages, and a blockade of a specific isoform was able to inhibit retinal leukostasis and BRB breakdown in both early and established diabetes (Ishida et al., 2003). $A_{2A}R$ activation is known to induce a release of VEGF and contribute to the detrimental effects of VEGF in pathological situations (Takagi et al., 1996; Liu et al., 2010), so it is possible that $A_{2A}R$ blockade could be ameliorating the effects of VEGF, and an assessment on VEGF levels in retinas treated with SCH 58261 would be useful.

All in all, this group of results shows that $A_{2A}R$ blockade is effective in improving RGC survival, and this may be the reason for the tentative improvement of the scotopic b-wave amplitude and scotopic OP2 and OP3 time to peak. It will be necessary to increase the number of animals used for these experiments to allow us to truly measure the potential of $A_{2A}R$ blockade in ameliorating the ERG dysfunctions provoked by diabetic conditions.

Chapter 6 General Discussion and Main Conclusions

6.1 General Discussion

Although many advances have been made in recent years towards effective therapeutics for diabetes, DR is still the most frequent cause of preventable blindness in working-age adults in the world (Yau et al., 2012). The progression and advance stages of DR have been extensively studied, and several mechanisms identified as therapeutic targets, while the variability of DR phenotype means that personalized medicine in DR is being increasingly sought, a method for relying on genomic biomarkers to predict the progression of DR patient-to-patient (Agarwal et al., 2014).

Most treatments are still focused on the vascular part of DR, and reducing vision loss: beyond laser and surgery treatments, several drugs targeting vitreous hemorrhage, as well as anti-VEGF therapies are proving to be effective in improving patients' vision (Titchenell and Antonetti, 2013). There are some therapies that attack the inflammatory state of DR, such as intravitreal implants of corticosteroids (Comyn et al., 2013) but, like the vast majority of strategies, are only recommended for more advanced stages, or when macular edema is detected. The pathogenesis of early DR is still a complex network of alterations that is only recently being truly explored in the search for molecular signals that may alert for the initiation of DR, as well as possible therapeutic targets.

The adenosinergic system is a complex signaling mechanism that spans a multitude of processes and functions, and is affected by various forms of stress or pathological conditions (Fredholm, 2007). Its four receptors can regulate a multitude of components, from other receptors to cytokines, chemokines and growth factors, and in turn be regulated by many signaling pathways (Dunwiddie and Masino, 2001; Sheth et al., 2014). The addition of these characteristics means that, in multifaceted DR, it becomes necessary to understand the alterations that occur in this system, for not only could they be directly involved in the pathology, or actively fight against it, but they could also interact or interfere with therapies used for diabetes and DR. Therefore, our main goal

was to understand the effects that DR has on the adenosinergic system, and then to probe the potential of adenosine and its receptors as a therapeutic target in the early stages of DR.

We have demonstrated that the adenosine receptors A_1R, $A_{2A}R$ and A_3R are altered in both retinal cell cultures under HG conditions and diabetic retinas. Since all three receptors have been implicated in inflammatory conditions (Haskó and Cronstein, 2004), while A_1R and $A_{2A}R$ are well know modulators of neurotransmission (Dunwiddie and Masino, 2001), these alterations may have an impact in the excitotoxic and inflammatory environment of DR. Adenosine removing enzymes ADA and AK have decreased expression in diabetic retinas at four weeks of diabetes, two alterations with a possible heavy impact on adenosine levels in the retina. If the same occurs in human diabetic retinas, it could explain the increased adenosine levels observed in diabetic patients with DR (Xia et al., 2014). Nucleoside transporters ENT1 and CNT2 are also altered, in cell cultures under HG conditions and diabetic retinas, respectively. The increase observed in the levels of CNT2 at four weeks of diabetes may be related to the decreased expression of AK and ADA at the same time point. If adenosine levels are affected by this, it is possible that transport through the gradient is ineffective, and an increase in CNT2 may indicate an attempt to circumvent the issue through the increase of active transportation of adenosine. It would be crucial to attempt to measure adenosine levels in the retina, either by the use of retinal explants or perhaps in the vitreous of diabetic animals, to further explore the impact of these alterations and possibly explore a link between them. Beyond this, the activity of the nucleotide degrading enzymes, NTPDases, is also affected by HG conditions, creating another possible influence to the increase in extracellular adenosine observed under the same conditions. It would be interesting to measure retinal NTPDase activity, possibly in retinal explants, to confirm that this alteration is also occurring in the diabetic retina.

Adenosine signaling is a transient and often sequential affair, a graceful process adapted for self-regulation. However, noxious conditions, and especially chronic

disturbances, can deregulate this, prolonging the effects of some ARs until they become nefarious, or activating unwanted signaling from others. Because the environment characteristic of DR involves several conditions affected by adenosine signaling, such as excitotoxicity, inflammation and angiogenesis, it is likely that the different ARs can be implicated in this, whether actively harmful, part of a protective feedback mechanism, or even a protective mechanism that becomes harmful due to over-activation, or interference by other aspects of the disease. All of this reinforces the need to understand the influence DR has on this system and vice-versa.

A_1R activation proved to be beneficial for cell survival under HG conditions, in a similar manner to $A_{2A}R$ blockade, in another demonstration of the conflicting roles of these two receptors, and likely through the inhibition of excessive glutamate release and amelioration of the toxic environment caused by HG conditions. The next step would be to explore this protective potential *in vivo*, in a method similar to the one used to investigate the effects of $A_{2A}R$ blockade, by treating diabetic animals with the A_1R agonist, and evaluating the effect on retinal function, RGC survival and inflammatory parameters. As for the protective effect witnessed upon ENT1 blockade, it would be necessary to confirm the hypothesis that its effects are occurring through A_1R activation, as we suspect, through a joint incubation with an A_1R antagonist.

The inhibition of $A_{2A}R$ signaling with SCH 58261 *in vivo* also proved to be protective, reducing the RGC loss that is characteristic of DR (Kern and Barber, 2008). The inability of $A_{2A}R$ blockade to decrease the expression of TNF and IL-1β hints that the mechanism behind this may be through management of excitotoxicity, or other mechanisms, and not exclusively by the control of inflammation. However, the low number of animals used and the inherent variation that comes with *in vivo* studies means that a increase in the number of these experiments is needed to verify this hypothesis.

The ERG recordings performed on control and diabetic animals were able to confirm that alterations to retinal function are already apparent at four weeks of diabetes.

Delays in the scotopic b-wave and OPs' time to peak, and also an increase in the scotopic b-wave amplitude are already detectable in this early stage of diabetes, most likely due to impaired function of third-order neurons, before the progression of retinal degeneration causes it to decrease. These recordings, as well as the recordings of control and diabetic animals treated with SCH 58261, were able to show that $A_{2A}R$ blockade is able to provide a recovery of normal b-wave amplitude for diabetic animals, and has the potential to reduce the delays of time to peak in scotopic OPs, for higher light intensities. On other parameters, such as photopic b-wave amplitudes, $A_{2A}R$ blockade had more modest, but still noticeable effects. The protective effects of $A_{2A}R$ blockade on RGC may help explain the inhibition of the increase in scotopic b-wave amplitude observed in non-treated diabetic animals, by protecting the feedback mechanisms generated by RGC, and perhaps amacrine cells as well. The effects of $A_{2A}R$ blockade on reducing time to peak delay of OPs suggest that this protective action is not restricted to RGCs, and an assessment of amacrine cell survival, as well as other cellular populations of the retina, is an essential future step. Not only it is important to investigate the potential neuroprotective effects of SCH 58261 in evaluating the survival of other neuronal populations, but it also is necessary to explore treatment methods beyond daily injections. A method that allows a continuous delivery of the drug over a period of time would be ideal, and there are implant devices already in use that are able to deliver a sustained release of the drug for months(Comyn et al., 2013). Also attractive for experimental purposes is the use of microspheres of biodegradable polylactide-co-glycolide that function as an implant (Herrero-Vanrell et al., 2014), and disappear from the site of administration after their time span of action.

In this work, we also explored the interactions of DPP4 and ADA in retinal cell cultures under HG conditions. We have shown that DPP4 can influence not only ADA activity but also the protein levels of this enzyme, an effect only partially abolished by an inhibitor of DPP4 enzymatic activity. Although it would be interesting to explore DPP4 activity in our cultures and the signaling mechanisms involved in the modulation of ADA

levels, as well as to study these interactions in the diabetic retina, the most interesting prospects lie in testing these interactions on human tissue. Due to the importance of DPP4-ADA binding on human cells, particularly immune cells, and the widespread use of DPP4 inhibitors in the treatment of diabetes, it becomes necessary to study the interaction of DPP4 with the adenosinergic system in human retinas and, in particular, whether this interaction is beneficial or detrimental in the context of DR.

6.2 Main Conclusions

Overall, the results presented in this thesis allowed us to draw the following conclusions:

- Elevated glucose and diabetic conditions induce alterations to the levels of the adenosine receptors A_1R, $A_{2A}R$ and A_3R in the retina. Elevated glucose increases ENT1 levels in retinal cells, while diabetic conditions increase CNT2 levels and decrease ADA and AK levels in the retina.
- Elevated glucose affects the activity of NTPDases, increasing the degradation of ATP/ADP, and affects the levels of intra and extracellular adenosine in retinal cell cultures.
- DPP4 is able to interact with ADA in rat retinal cells in a manner detectable by co-immunoprecipitation. DPP4 incubation increases ADA activity and protein levels in HG conditions. Inhibition of DPP4 activity annuls the increased levels of ADA, but only partially reverts the effect on ADA activity, suggesting that DPP4 is affecting ADA activity in a manner unrelated with enzymatic activity.
- A_1R activation, $A_{2A}R$ antagonism, and ENT1 blockade are able to decrease the cell death induced by elevated glucose in retinal cell cultures.

- $A_{2A}R$ blockade in diabetic animals prevented the alterations in the scotopic b-wave induced by diabetic conditions, and reduced delays in time to peak of scotopic OP2 and OP3.
- $A_{2A}R$ blockade in diabetic animals increased RGC survival in diabetic retinas.

Taken together, these results describe the extent of the impact of elevated glucose and diabetic conditions on the different components of the adenosinergic system, from receptors, to enzymes and transporters, and theses alterations may help shape a valuable insight into the role of adenosine in the diabetic retina. The modulation of this system was shown to be able to protect retinal cells from the increased cell death induced by elevated glucose, and $A_{2A}R$ blockade not only protected RGCs, but was also able to revert some of the alterations in retinal function caused by diabetes, revealing a potential to improve retinal function in diabetic retinas. It is possible that the elevated adenosine levels in retinal cultures under HG conditions, coupled with the increased levels of $A_{2A}R$, will potentiate excitatory $A_{2A}R$ signaling and, in an environment where glutamate metabolism is already affected, an increase in $A_{2A}R$ signaling will compound on the severity of the situation, by increasing the release and inhibiting the uptake of glutamate, becoming a player in excitotoxic neurodegeneration. Furthermore, the fact that $A_{2A}R$ signaling does not disturb microglial functions in their quiescent state, while it does have pro-inflammatory actions upon activation means that, again, $A_{2A}R$ signaling is being potentiated at the worst time, with levels of both receptor and adenosine rising at the same time diabetic or HG conditions trigger microglial activation.

The results presented in this work suggest that the modulation of the adenosinergic system, and particularly the $A_{2A}R$, has the potential to be a suitable pharmacological strategy for prevention/treatment of neuronal cell death and vision loss among diabetics, targeting diabetic retinopathy in its early stages, and improving the quality of life for millions of people around the world.

Chapter 7 References

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