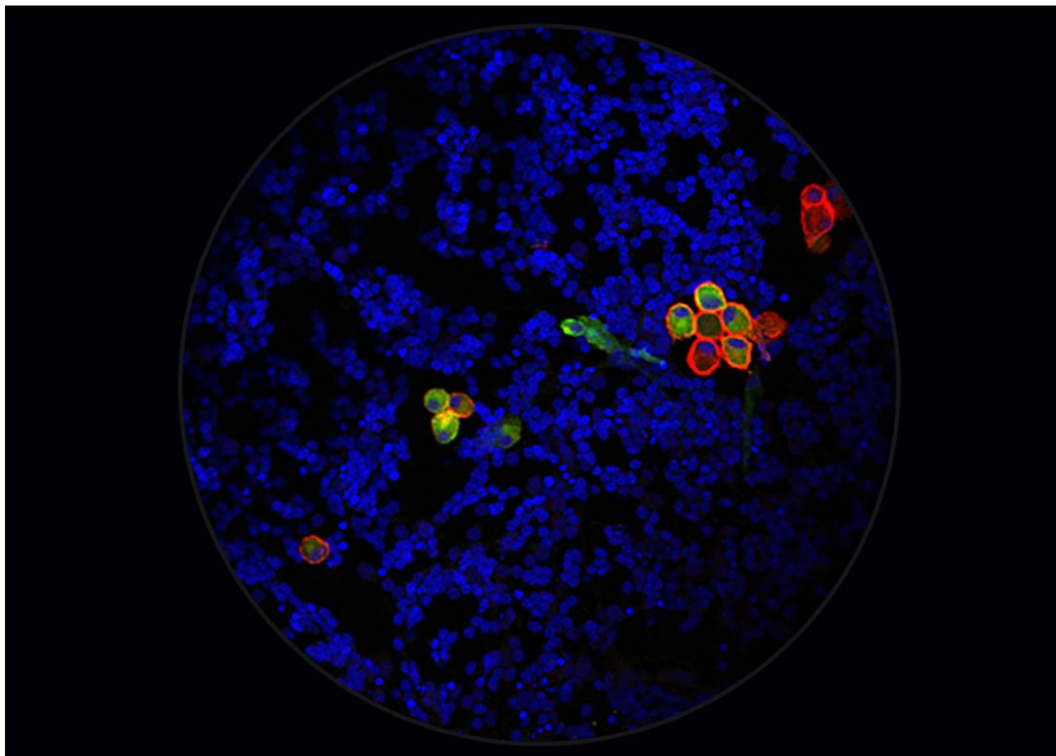


Sandra Raquel Félix Guimarães Correia

Involvement of the NPY system in the inflammatory responses mediated by retinal microglial cells: modulation by sitagliptin

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

Universidade de Coimbra

On the front page:

Primary retinal neural cell culture stained with CD11b (red) and iNOS (green). Cell nuclei stained with DAPI (blue).

Involvement of the NPY system in the inflammatory responses mediated by retinal microglial cells: modulation by sitagliptin

Sandra Raquel Félix Guimarães Correia

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

Universidade de Coimbra

2014

Execution of this work was supported by:

FCT (PTDC/NEU-OSD/1113/2012) and Strategic Project (PEst-C/SAULA0001/2013-2014) and COMPETE-FEDER; Portugal.

“You can be anything you want to be.

Just turn yourself into anything you think that you could ever be”

Queen in Innuendo

Agradecimentos

Começo por agradecer ao Doutor Francisco Ambrósio, ao Doutor Flávio Reis e à Doutora Ana Paula Silva por terem permitido que conjugasse o meu trabalho no LCEA com estes 2 anos de mestrado, sem nunca duvidarem de que eu seria capaz de fazer ambas as coisas.

Agradeço ao Doutor Francisco Ambrósio, orientador desta tese, por me ter aceitado pela segunda vez para desenvolver um trabalho científico no seu grupo de investigação. Obrigada pela orientação, pela confiança depositada em mim e por nunca terem faltado recursos para o desenvolvimento deste trabalho. Agradeço também toda a partilha de conhecimento que contribuiu para o meu desenvolvimento pessoal e académico.

À Doutora Raquel Santiago, agradeço a transmissão de conhecimento e todo apoio e incentivo que me foi dando ao longo deste ano. Demonstrando amizade, mas sempre de forma exigente, considero que o seu trabalho como co-orientadora foi muito importante para que eu conseguisse desenvolver esta tese.

Agradeço também à Professora Doutora Manuela Grazina por ter autorizado a utilização do Real-Time-PCR e à Maria João pela paciência e por me ter ajudado.

Agradeço a todos os meus colegas de laboratório que tornam o trabalho mais simples. Pela boa disposição, pelo bom ambiente e porque nunca me faltou ajuda quando precisei. Obrigada por terem tratado dos ratos nesta última fase. Com o passar dos anos (sim, porque já lá vão 3!) a minha opinião sobre este grupo só melhora! Obrigada Raquel, Inês, Joana Martins, Filipa, João, Maria, Marlene, Tânia, Dan, Elisa, Catarina Neves, Joana Horta, Sónia, Tiago, Pedro, Filipe, Joana Vindeirinho, Catarina Gomes, Paulo Santos e António. Agradeço também à Carla Marques pelo apoio e companheirismo.

Obrigada à Maria, por me ter acompanhado no início deste trabalho, ensinando-me muito do que aprendi durante este ano. Obrigada também pelo apoio.

Agradeço à Fi por ser uma espécie de irmã mais velha, não só para mim, mas para todo o grupo.

À Joana Martins, minha amiga e colega de gabinete, por ter acompanhado todo o meu trabalho (até ao fim!), e pela amizade.

À Inês porque fizemos isto juntas! Por me ter acalmado nos momentos de desespero. E por me fazer rir das coisas sérias.

Faço questão de agradecer em particular ao João. Por me ter ajudado durante o ano no laboratório e porque nesta fase final me surpreendeu, mostrando-se sempre disponível, apesar de também ter uma tese para escrever. E sempre nas calmas... Tenho muito a aprender contigo!

À Raquel Boia, porque se revelou uma óptima professora e, muito mais importante do que isso, uma grande amiga. A disponibilidade para ajudar e a motivação foram muito importantes para que conseguisse terminar este trabalho. Obrigada por todas as "murraças" e por teres acreditado na minha capacidade (às vezes mais do que eu). Afinal era possível!!

À Marau, porque só nos conhecemos há uns mesitos mas a relação de amizade foi quase automática. Obrigada por toda a ajuda, todos os "não te preocupes com os ratos agora!".

Agradeço aos meus amigos de Espinho, em particular às Dianas e à Joana, que estiveram presentes, mesmo à distância e me deram motivação extra nesta fase final.

Ao meu pai e aos meus avós, agradeço todo o apoio e preocupação. Porque sempre que me queixei do excesso de trabalho, a resposta da minha avó foi "ainda bem! Sem trabalho nada se consegue!". É verdade.

Agradeço à minha mãe porque é muito bom sentir todo o amor, carinho e preocupação de mãe. Porque nunca me falhou. E por ser, sem dúvida, a pessoa mais forte que eu conheço, sendo uma inspiração para mim sempre que os obstáculos são difíceis de ultrapassar.

Agradeço às minhas irmãs porque agora que já não temos idade para andar à porrada, vocês tornaram-se nas minhas melhores amigas. E esta é uma daquelas coisas que tenho a certeza que é para sempre.

Ao meu principezinho Tomás, que é um motivo de alegria pura na minha vida. Porque todos os problemas se desvanecem quando estou com ele.

Agradeço sobretudo ao Filipe, por ter estado sempre ao meu lado. Por aturar todos os meus dramas, stresses e ansiedades, sem nunca deixar de me apoiar. Por toda a paciência e motivação que me deu para que eu conseguisse atingir mais um objectivo importante na minha vida. E também por ter mostrado sempre interesse pelo meu trabalho e ter acompanhado todos os pormenores deste percurso. Obrigada por tudo.

Eu pensava que não ia escrever muito nesta secção, mas é muito bom parar para pensar que tenho ao meu lado tantas pessoas a quem agradecer. Obrigada a todos.

Contents

Abbreviations iii
Resumo v
Abstract vii
1. Introduction 3
1.1. Eye 3
1.2. Retina 4
1.2.1. The Neuronal Retina 6
1.2.2. Glial cells of the retina 8
1.3. Diabetic retinopathy 11
1.4. Diabetic retinopathy and inflammation 12
1.5. Neuropeptide Y 13
1.5.1. Biosynthesis and metabolism 14
1.5.2. NPY Receptors 15
1.6. Neuropeptide Y system in the retina 16
1.7. Neuropeptide Y and microglia 17
1.8. Sitagliptin and DPP-IV 17
Objectives 19
2. Methods 21
2.1. Animals 21
2.2. Primary Retinal Neural Cell Cultures 21
2.3. Retinal Organotypic Cultures 21
2.4. Drug Exposure 22
2.5. Immunocytochemistry 23
2.6. Immunohistochemistry 23
2.7. Image Analysis 24
2.8. Nitrite Quantification Assay 25
2.9. Enzyme-Linked Immunosorbent Assay 25
2.10. RNA Extraction 26
2.11. cDNA Synthesis 26
2.12. Real-Time Quantitative PCR 27
2.13. qRT-PCR data analysis 27
2.14. Statistical Analysis 28
3. Results 31

3.1. Sitagliptin reduces LPS-triggered increase in iNOS immunoreactivity and NO production in microglia in primary retinal neural cell cultures ... 31

3.2. Effect of sitagliptin and Y_1 receptor blockade in LPS-induced increase in cytokine production in primary retinal neural cell cultures ... 34

3.3. Sitagliptin prevents LPS-induced alterations in microglia morphology in cultured retinal explants ... 38

3.4. Sitagliptin reduces the increase in iNOS immunoreactivity in microglial cells triggered by LPS in cultured retinal explants ... 41

4. Discussion ... 47

5. Conclusion ... 55

6. References ... 59

Abbreviations

AGEs	Accelerated formation of glycation end products
BRB	Blood-retinal barrier
CNS	Central Nervous System
CPON	C-terminal peptide of NPY
Ct	Cycle threshold
CTCF	Corrected total cell fluorescence
DAPI	4',6-diamidino-2-phenylindole
DIV	Days *in vitro*
DMEM/F-12	Dulbecco's modified Eagle medium
Dnase I	Deoxyribonuclease I
DPP-IV	Dipeptidyl-peptidase-IV
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GCL	Ganglion cell layer
GFAP	Glial fribrillary acidic protein
GLP-1	Glucagon-like peptide-1
HBSS	Hanks balanced salt solution
ICAM-1	Intracellular adhesion molecule-1
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IL-1β-IR	IL-1β immunoreactivity
INL	Inner nuclear layer
iNos	Inducible nitric oxide synthase
iNOS-IR	iNOS immunoreactivity
IPL	Inner plexiform layer
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MEM	Eagle's minimum essential medium
NE	Norepinephrine
NF-κB	Nuclear factor-kB
NGS	Normal goat serum

NO	Nitric oxide
NPY	Neuropeptide Y
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKCζ	Protein kinase C
PP	Pancreatic polypeptide
PYY	Peptide tyrosine-tyrosine
RGCs	Retinal ganglion cells
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Room temperature
TE	Tris-EDTA
TLR4	Toll-like receptors 4
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Resumo

A retinopatia diabética é uma das principais complicações da diabetes, sendo também uma das principais causas de perda de visão e cegueira. O controlo eficaz da glicémia, dislipidémia e pressão arterial podem reduzir significativamente o risco de desenvolver esta doença. Além disso, as opções terapêuticas disponíveis atualmente são limitadas e invasivas, e no essencial, são utilizadas nas fases mais avançadas da doença. Deste modo, é prioritário desenvolver novas estratégias terapêuticas para a prevenção e tratamento da retinopatia diabética.

Os processos inflamatórios têm sido apontados como tendo um papel importante na patogénese da retinopatia diabética. Foi demonstrado que vários processos inflamatórios, tais como o aumento dos níveis de monóxido de azoto (NO) e de citocinas pró-inflamatórias, e também a ativação das células da microglia, da via do NF-κB e da PKCζ estão relacionados com a rutura da barreira hemato-retiniana.

A sitagliptina é um inibidor da dipeptidil peptidase 4 (DPP-IV) que é utilizada no tratamento da diabetes tipo 2. Resultados recentes revelaram que a sitagliptina previne a rutura da barreira hemato-retiniana e também o aumento dos níveis de interleucina (IL)-1β em retinas de animais com diabetes tipo 1 e tipo 2.

Além do seu efeito na estimulação da secreção de insulina, a DPP-IV tem um papel importante no metabolismo do neuropeptídeo Y (NPY). Assim, a inibição desta enzima pode afetar as funções moduladoras do NPY.

Neste estudo, pretendeu-se avaliar se a sitagliptina inibe a ativação das células da microglia da retina e a neuroinflamação, e se os efeitos deste fármaco são mediados pelo sistema do NPY, e em particular pela ativação dos recetores Y_1.

Os efeitos da sitagliptina foram avaliados em culturas primárias de retina e em culturas organotípicas de retina. Para induzir uma resposta inflamatória, ambas as culturas foram expostas ao lipopolissacarídeo (LPS) e os efeitos da sitagliptina foram testados, na ausência e na presença do antagonista dos receptores Y_1 (BIBP3226).

Nas culturas primárias de retina, a sitagliptina inibiu o aumento da imunoreactividade da isoforma indutível da sintase do monóxido de azoto (iNOS-IR) nas células da microglia e também o aumento da produção de NO

induzidos por exposição a LPS. O bloqueio dos receptores Y_1 inibiu parcialmente, mas não significativamente, o efeito da sitagliptina no aumento da iNOS-IR. No entanto, o antagonista do recetor Y_1, BIBP3226, preveniu completamente o efeito da sitagliptina na produção de NO. Estes resultados sugerem que os efeitos da sitagliptina na inflamação podem ser mediados, pelo menos em parte e, sob determinadas condições, pela ativação dos recetores Y_1. Os níveis do fator de necrose tumoral (TNF) e de IL-1β nas culturas primárias de retina não foram afetados pelo tratamento com sitagliptina nem pelo bloqueio dos recetores Y_1. Contudo, resultados preliminares indicam que a sitagliptina atenua o aumento da imunoreactividade da IL-1β em células da microglia, induzido pelo LPS.

Nas culturas organotípicas de retina, a sitagliptina preveniu as alterações na morfologia das células da microglia. Apesar de não ser significativo, este efeito foi parcialmente inibido pelo bloqueio dos recetores Y_1, de modo semelhante ao que se tinha sido observado nas experiências em que se avaliou iNOS-IR em culturas primárias de retina, sugerindo que os efeitos da sitagliptina na morfologia das células da microglia não parecem depender da ativação dos recetores Y_1. À semelhança do que foi observado nas culturas primárias de retina, a sitagliptina também inibiu o aumento da iNOS-IR em células da microglia nas culturas organotípicas. Mais uma vez, o bloqueio dos recetores Y_1 inibiu, mas de forma parcial e não significativa, o efeito da sitagliptina. Por último, a sitagliptina não inibiu o aumento da expressão de mRNA da iNOS, induzido pelo LPS, mas o bloqueio dos recetores Y_1 exacerbou o aumento da expressão de mRNA da iNOS induzido por LPS.

Concluindo, estes resultados indicam que a sitagliptina tem efeitos anti-inflamatórios, controlando a reatividade das células da microglia, mas estes efeitos parecem ser mediados por vias de sinalização específicas, como por exemplo a da iNOS. Apesar dos resultados sugerirem que o sistema do NPY, e em particular o recetor $Y_{1,}$ poderá contribuir parcialmente para alguns dos efeitos da sitagliptina, serão necessários mais estudos para clarificar se a activação dos recetores Y_1 está efetivamente a mediar os efeitos da sitagliptina.

Palavras-chave: células da microglia, neuroinflamação, sitagliptina, recetores Y_1.

Abstract

Diabetic retinopathy is one of the most common complications of diabetes and is a leading cause of vision loss and blindness. Intensive glycemic control, blood pressure regulation and lipid-lowering therapy reduce the risk of developing this disease. Moreover, there are significant limitations in the therapeutic approaches available, which are invasive and mainly targeted for the later stages of the disease. Therefore, there is a great need to develop new therapeutic strategies for prevention and treatment of diabetic retinopathy.

It has been shown that low-grade inflammatory processes play an important role in the pathogenesis of diabetic retinopathy. Moreover, it has been reported that the blood-retinal barrier (BRB) breakdown is correlated with increased nitric oxide (NO) production in the retina, mainly via inducible nitric oxide synthase (iNOS), microglia activation, increased levels of pro-inflammatory cytokines, and nuclear factor-kB (NF-κB) and protein kinase C (PKCζ) activation.

Sitagliptin is an inhibitor of dipeptidyl-peptidase-IV (DPP-IV) used for the treatment of type 2 diabetes. Recent published data has shown that sitagliptin can prevent the BRB breakdown. Additionally, it prevented the increase of interleukin (IL)-1β in the retinas of type 1 and type 2 diabetic animals.

Besides stimulating insulin secretion, the inhibition of DPP-IV also affects the modulatory functions of neuropeptide Y (NPY), because DPP-IV is a key enzyme on the metabolism of NPY.

In this study, we aimed to investigate whether sitagliptin is able to inhibit retinal microglia activation and neuroinflammation and whether the effects of sitagliptin are mediated by the NPY system, and particularly through Y_1 receptor activation. Using primary retinal neural cell cultures and retinal organotypic cultures exposed to lipopolysaccharide (LPS) to trigger an inflammatory response, we evaluated the effects of sitagliptin in microglial reactivity, in the absence or presence of Y_1 receptor antagonist (BIBP3226).

In primary retinal neural cell cultures, sitagliptin was able to inhibit the increase in iNOS immunoreactivity (iNOS-IR) in microglial cells and NO production triggered by LPS. The blockade of Y_1 receptor partially inhibited the effect of sitagliptin on the increase of iNOS-IR triggered by LPS, although not significantly. However, the Y_1 receptor antagonist, BIBP3226, completely

abolished the effect of sitagliptin on NO production, suggesting that Y_1 receptor might mediate, at least in part, and under certain condition, the effect of sitagliptin on inflammation. Moreover, neither sitagliptin nor BIBP3226 affected tumor necrosis factor (TNF) or IL-1β levels in retinal neural cell cultures. However, preliminary results show that sitagliptin seemed to attenuate LPS-induced upregulation of IL-1β immunoreactivity (IL-1β-IR) in microglial cells.

In cultured retinal explants, sitagliptin prevented the alterations in retinal microglia morphology. The blockade of Y_1 receptor by BIBP3226 slightly inhibited the effects of sitagliptin, although not significantly, similarly as was observed in the experiments where iNOS-IR was evaluated in retinal neural cell cultures, suggesting that the effects of sitagliptin on microglia morphology appear to be not dependent of the activation of Y_1 receptor. As in retinal neural cell cultures, sitagliptin also inhibited the increase in iNOS-IR in microglial cells in cultured retinal explants, and again, despite a tendency for a partial inhibition of the effect of sitagliptin when cultures were also exposed to the Y_1 receptor antagonist, this effect was not statistically significant. Finally, sitagliptin did not inhibit the increase in iNOS mRNA expression triggered by LPS, but the blockade of Y_1 receptor enhanced the increase in iNOS mRNA expression induced by LPS.

In conclusion, these results clearly indicate that sitagliptin has anti-inflammatory effects by controlling the reactivity of microglial cells, but these effects of sitagliptin appear to affect specific targets or pathways, such as iNOS. Moreover, it seems that the NPY system, and particularly the Y_1 receptor, might eventually partially contribute for some of the effects of sitagliptin, but further investigation is required to clarify whether Y_1 receptor activation mediates the effects of sitagliptin.

Key-words: Microglial cells, neuroinflammation, sitagliptin, Y_1 receptor.

Chapter 1

Introduction

1. Introduction

1.1. Eye

The eye is a very complex and highly developed structure that provides the sense of sight. This organ is capable of capturing incoming light and converting it into electrical signals that are processed and refined by our brain, which translates them into visual images (Purves, 2004). It is exceptional that visual information can be discerned over a wide range of stimulus intensities that allows us to distinguish shapes, colors, size and textures.

The eyeball can be divided in three main layers: the outer, the middle and the inner layer.

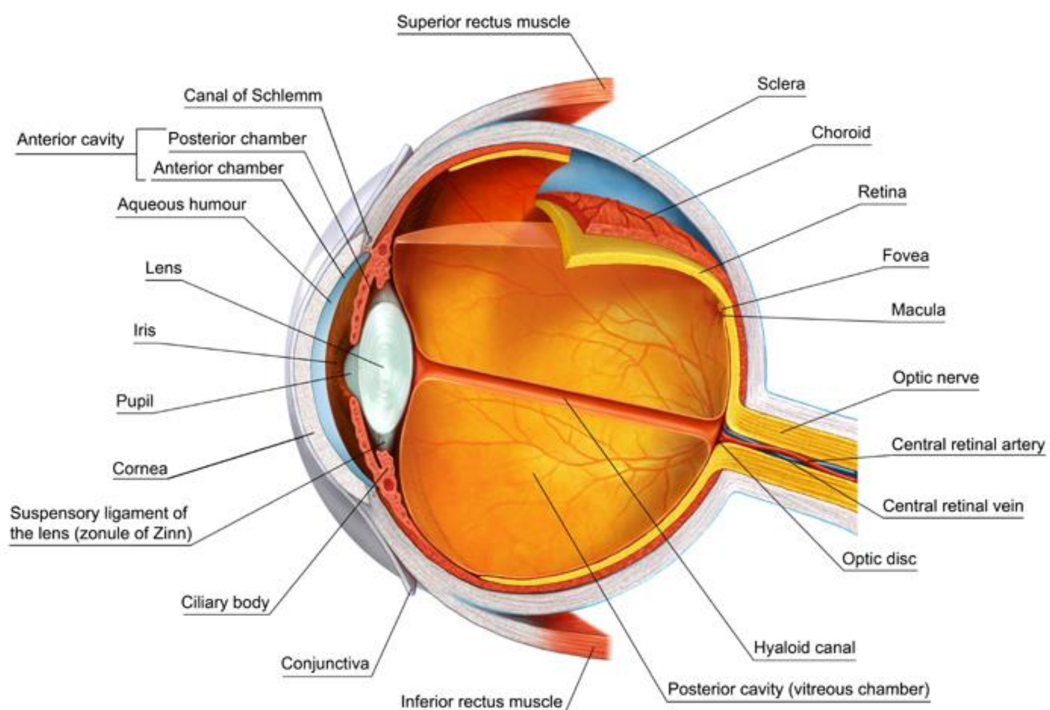


Fig. 1 - Anatomy of the human eye (from www.myvmc.com).

The outer layer is composed by the sclera and the cornea. The sclera is a tough white, opaque, fibrous tissue that helps maintaining the eye form and protects its internal structures. At the front of the eye, in the area protected by the eyelids, the sclera is transformed into the cornea, a specialized, transparent tissue through which light rays enter the eye. This is the most powerful lens of the optical system and along with the crystalline lens, allows the production of a sharp image in the retina (Fig. 1) (Purves, 2004)

The iris, ciliary body and the choroid form the uvea, which is the middle layer of the eye. The largest component of the uveal tract is the choroid that is formed

by a rich capillary bed that nourishes the photoreceptors in the outer retina. The choroid also has a high concentration of melanin that absorbs light, controlling reflection within the eye. The ciliary body is a muscular component that encircles the lens and changes its shape during the process of focusing. This part of the uvea also comprises a vascular component, the ciliary processes, which produce the fluid that fills the front of the eye: the aqueous humor. The iris is the circular, colored portion of the eye that surrounds the pupil. It contains two sets of muscles with opposing actions that allow the pupil to dilate or constrict, regulating the amount of light that enters the eye (Kolb, 1995; Purves, 2004).

The retina is the innermost layer of the eye and contains, among other cell types, light-sensitive photoreceptors capable of transforming light into electrical signals that are sent to the brain through the optic nerve (Purves, 2004).

The eye also comprises three different fluid chambers: the anterior chamber that lies behind the cornea and in front of the lens, the posterior chamber located between the iris and the lens, and the vitreous chamber that extends from the lens to the retina. The first two chambers are filled with aqueous humor, a watery liquid that supplies nutrients and oxygen to the cornea and lens. It is constantly produced by the ciliary processes and flows into the anterior chamber through the pupil, being continually drained through the trabecular meshwork. To maintain a constant intraocular pressure, it is important that the rates of production and drainage of this fluid are balanced. In the vitreous chamber lies a thicker, gelatinous substance called the vitreous humor that represents about 80% of the volume of the eye. Besides maintaining the shape of the eye, this fluid contains phagocytic cells that remove unwanted debris in the visual field (Kolb, 1995; Purves, 2004).

1.2. Retina

The retina is a light-sensitive neural tissue, approximately 0.5 mm thick in humans that lines the back of the eye (Kolb, 1995). When light strikes the retina, it is absorbed by the photoreceptors, where electrochemical signals are created and sent to the brain via axons into the optic nerve. The optic nerve is formed by axons of retinal ganglion cells (RGCs) and blood vessels that open into the retina to vascularize the inner layers of this tissue.

Being part of the central nervous system (CNS), the retina comprises five types of neurons: photoreceptors, bipolar cells, horizontal cells, amacrine cells and RGCs. These cells are highly organized in three layers of nerve cell bodies and two layers of synapses (Fig.2). The outer nuclear layer (ONL) is the outermost layer of the retina and contains cell bodies of the photoreceptor cells, rods and cones. Cell bodies of the bipolar, horizontal and amacrine cells are located in the inner nuclear layer (INL) and the ganglion cell layer (GCL) is composed by cell bodies of RGCs and displaced amacrine cells (Kolb, 1995).

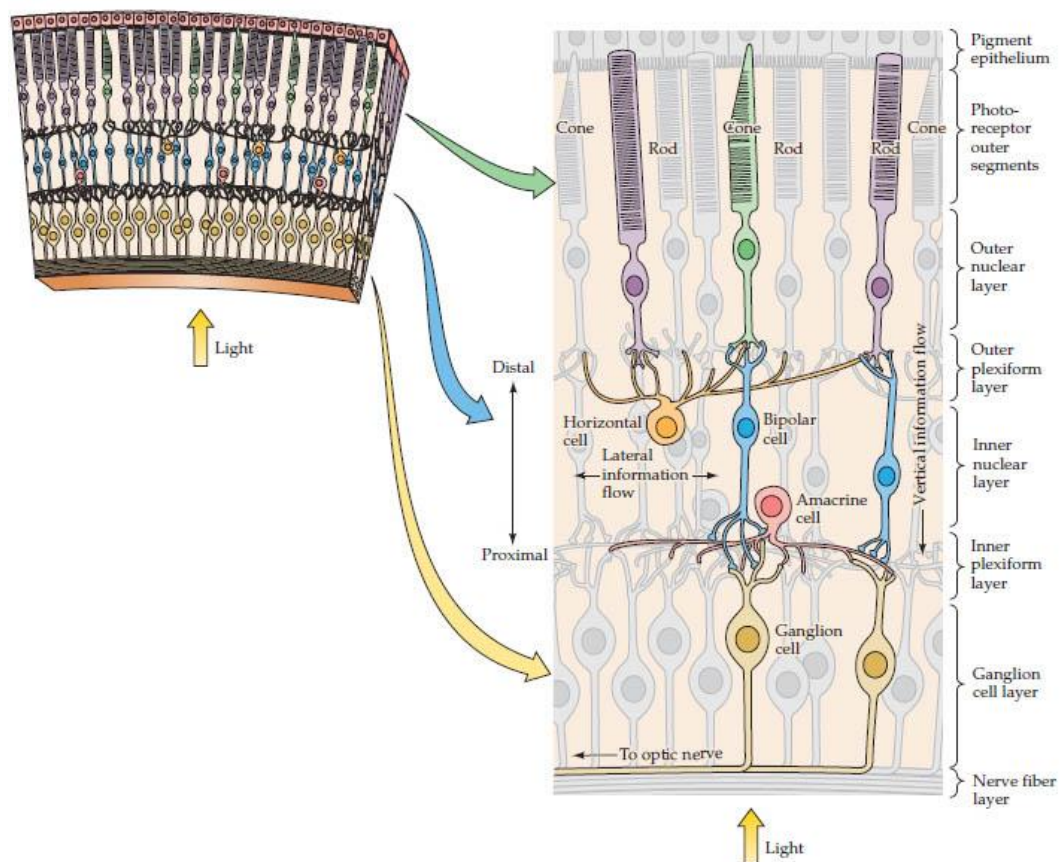


Fig. 2 – Schematic illustration of the structure of the retina (adapted from Purves, 2004).

Between the GCL and the INL lies the inner plexiform layer (IPL), where the bipolar cells have their axon terminals and synapse with the dendrites of RGCs and amacrine cells (Fig.2). The other synaptic layer is called outer plexiform layer (OPL) and is located between the INL and the ONL. This part of the retina is where connections between photoreceptors and bipolar and horizontal cells take place. The retina is separated from the choroid by the retinal pigment epithelium (RPE), a layer of pigmented cuboidal cells that, among other functions, controls the access of nutrients from the choroid to the retina,

phagocytizes the outer segments of photoreceptors and contributes to the normal retinal immune regulation, secreting cytokines (Amirpour et al., 2014; Langmann, 2007). In addition to neurons, there are several other types of cells in the retina, which include glial cells, endothelial cells, pericytes and epithelial cells.

While choroidal blood vessels supply photoreceptors, the inner retina is nourished by the retinal artery that branches into three capillary networks throughout the retina. Between endothelial cells and retinal capillaries there are tight junctions, which are also present in epithelial cells of the RPE. The RPE, along with retinal capillaries constitute the blood-retinal barrier (BRB) (Fig.3). The inner BRB is composed by retinal capillaries, while the outer BRB is formed by the RPE. By regulating the transport of molecules into the retina, the BRB plays a very important role in protecting the retina against circulating toxins (Siu et al., 2008).

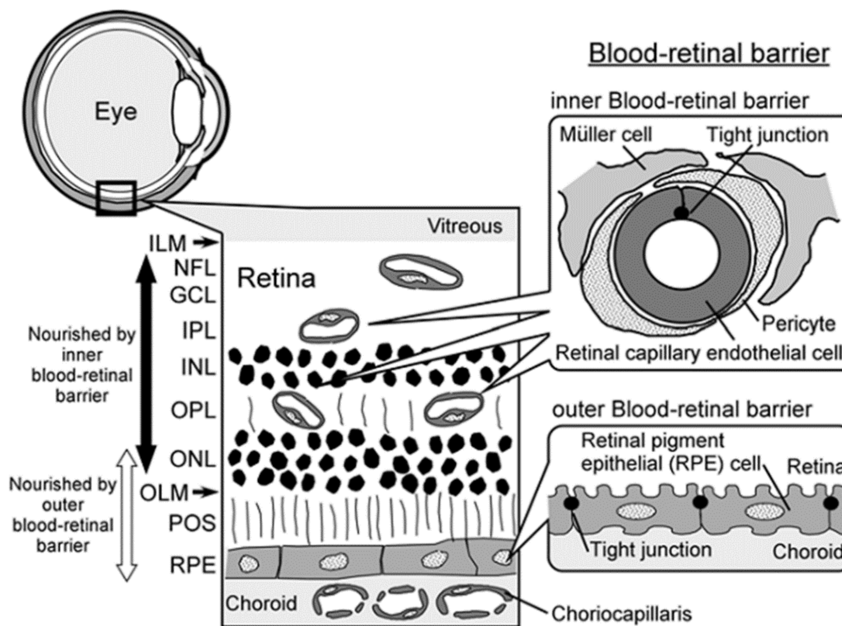


Fig. 3 - Schematic diagram of the blood-retinal barrier. RPE: retinal pigment epithelium; POS: photoreceptor outer segments; OLM: outer limiting “membrane”; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; NFL: nerve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

1.2.1. The Neuronal Retina

1.2.1.1. Photoreceptors

There are two types of photoreceptors: rods and cones. In the human retina, there are about 110-125 million rods. Functionally, these cells are specialized to

yield high sensitivity, permitting twilight and night vision. On the other hand, the 6-7 million cones are responsible for high resolution and color perception (Lang, 2000). Both have an outer segment, which contains light-sensitive photopigments and an inner segment, where the cell nucleus, mitochondria and synaptic terminals are located, and contact with bipolar and horizontal cells. Photoreceptors absorb light by the photopigment in the outer segment and this initiates a cascade of processes that change the membrane potential, and the amount of neurotransmitter released by the photoreceptor that synapses with horizontal and bipolar cells (Purves, 2004).

The distribution of rods and cones across the retina is very important for vision. The density of rods throughout most of the retina is much greater than cones. However, in the fovea (a highly specialized region of the central retina), cone density increases almost 200-fold. This extremely higher density of cone receptors in the fovea provides high visual acuity (Purves, 2004).

1.2.1.2. Bipolar Cells

Bipolar cells transmit the synaptic input received from photoreceptors to ganglion and amacrine cells. There is only one type of bipolar cells that receives inputs from rods and they are called rod bipolar cells. On the other hand, there are ten different types of bipolar cells that synapse with cones and these cells are designated cone bipolar cells (Kolb, 1995). Cone bipolar cells can be classified as ON bipolar cells and OFF bipolar cells, depending on their response to glutamate released by photoreceptors. OFF bipolar cells have excitatory ionotropic glutamate receptors and are hyperpolarized by light. ON bipolar cells express metabotropic glutamate receptors and are depolarized by light (Fischbarg, 2006).

1.2.1.3. Retinal Ganglion Cells

Retinal ganglion cells are located in the innermost layer of the retina, being the final output neurons of this tissue. These cells collect visual signals from bipolar and amacrine cells and transmit them through axons to the brain. According to the pathways initiated by bipolar cells, RGCs can also be divided into ON and OFF (Kolb, 1995).

1.2.1.4. Horizontal cells

Horizontal cells are connected laterally to many rods, cones, and bipolar cells. Their processes enable lateral interactions between these neighboring cells, maintaining the visual system sensitivity by selectively suppressing certain nerve signals. This process is called lateral inhibition and improves the contrast and definition of the visual stimulus (Kolb, 1995; Purves, 2004).

1.2.1.5. Amacrine cells

Amacrine cells are interneurons that integrate, modulate and interpose a temporal domain to the visual message presented to the RGCs. Synaptically active in the IPL, they can be classified into more than 20 subtypes and help to extract visual elements, such as contrast, color, brightness and movement (Kaneda, 2013; Kolb, 1995).

1.2.2. Glial cells of the retina

1.2.2.1. Müller cells

Müller cells are the most abundant glial cells in the retina and span the entire depth of this tissue, forming architectural support structures. They play an essential role in the normal function of retina by regulating extracellular concentrations of potassium, which are increased upon light stimulation. In addition, Müller cells remove neurotransmitters, such as glutamate, from extracellular space following their release into the synapse (Newman and Reichenbach, 1996).

1.2.2.2. Astrocytes

Astrocytes exert several essential functions related to the CNS homeostasis. In response to insults to the CNS, these cells proliferate, change their morphology and increase the expression of glial fribrillary acidic protein (GFAP), in a process called astrogliosis. Astrocytes are part of the BRB, having a close association with retinal vessels and regulating its properties. Additionally, they form a nutritive support in providing glucose to neurons (Kolb, 1995).

1.2.2.3. Microglia

Microglial cells play an important role in the surveillance of the CNS microenvironment. In healthy conditions, microglia presents a ramified morphology extending long thin processes. Upon infection, trauma, ischemia and neurodegenerative disease, these cells become activated and with increased phagocytic capacity. They migrate to the site of injury, proliferate and release a variety of factors, such as cytokines, nitric oxide (NO) and reactive oxygen species (ROS) (Kettenmann et al., 2011).

Microglial cells are sensitive to pro-inflammatory mediators through surface molecules, such as cytokine receptors, scavenger receptors, pattern recognition receptors and chemokine receptors. The activation of these receptors leads to increased microglial activation, which contributes to the ongoing inflammation process (Kierdorf and Prinz, 2013). In neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, chronic activation of microglia contributes to the progression of the disease.

1.2.2.4. Microglia in the retina

In the retina, under physiological conditions, microglial cells are found in a non-activated state in the NFL, GCL and IPL (Fig. 4) (Santiago et al., 2014).

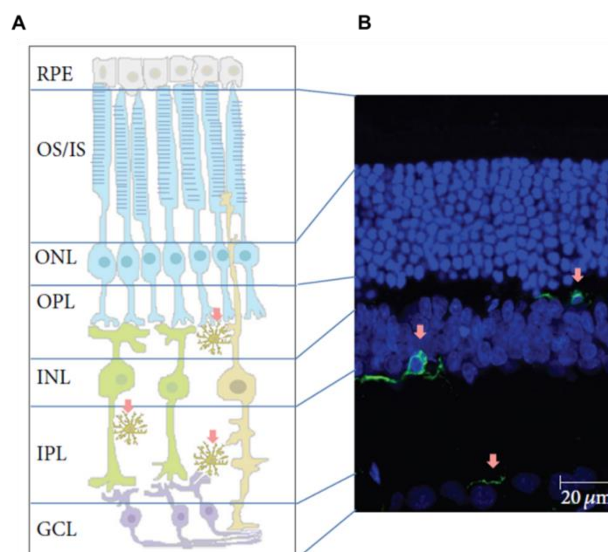


Fig. 4 - Schematic draw of microglia localization in the retina. Microglial cells (pink arrows) in normal physiological conditions are mainly located in the plexiform layers. (A) Retinal layers: OS/IS, outer and inner segments of rods and cones; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (B) Confocal image from a rat retinal section (Santiago et al., 2014).

As mentioned previously, in their resting state, microglial cells present highly motile protrusions and extensive processes that continuously scan their environment. They phagocytize cell debris and release mediators that regulate the survival of RGCs and photoreceptors (Langmann, 2007).

In response to injury, microglial cells become activated and migrate throughout the retina to the site of lesion where they alter their morphology, becoming amoeboid-shaped cells with the ability to proliferate and phagocytize cellular debris and apoptotic cells (Fig. 5) (Garden and Moller, 2006).

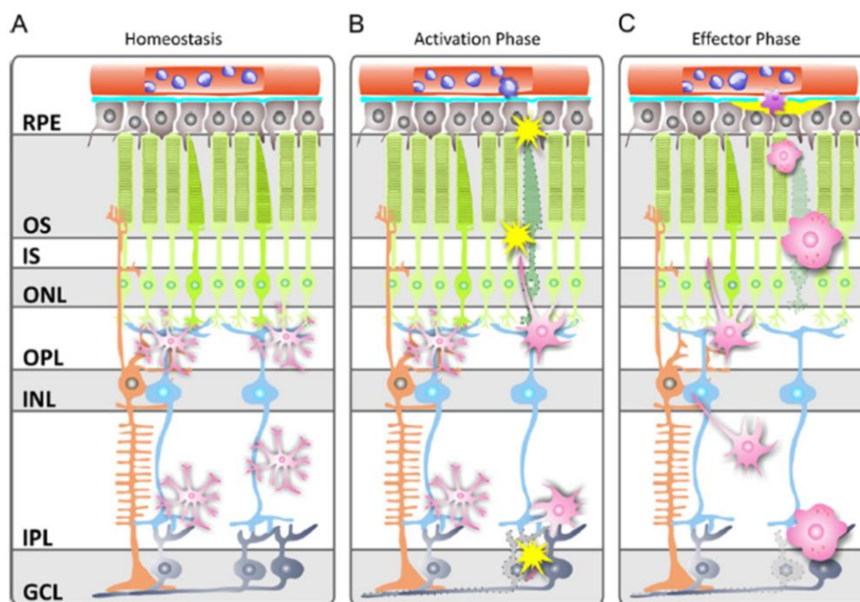


Fig. 5 - Schematic representation of microglia activation and migration in the retina. (A) In homeostatic conditions microglia are located mainly in the plexiform layers of the retina, scannig their environment, phagocytosing cell debris and secreting neurotrophins; (B) Stimulus that lead to abnormal cell functions are sensed by microglia; (C) Microglia migrates to the injury site, alter their morphology into an amoeboid-shape and become able to phagocytize. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer (Karlstetter et al., 2010).

In this activated state, microglia produce and release pro-inflammatory mediators, such as nitric oxide, reactive oxygen species and cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1β and IL-6. In addition, microglial cells release chemokines that attract other immune cells and secrete several neurotrophic factors, influencing the physiology and survival of surrounding neurons (Kettenmann et al., 2011).

When activated, microglial cells can be either beneficial or harmful, depending on their immunological phenotype and the local cytokine milieu. Chronic

activation leads to exaggerated responses that can induce retinal damage and neuronal apoptosis (Langmann, 2007). In fact, studies reveal that microglia is activated in an early stage of retinal disease, suggesting that these cells have an inductive role in disease progression (Lee et al., 2008).

1.3. Diabetic retinopathy

Diabetic retinopathy is one of the most common complications of diabetes and is the leading cause of preventable blindness in working-age adults (Fong et al., 2004). Nearly all individuals who have had diabetes for more than 15 years develop the disease to some degree, regardless of whether they have type 1 (due to loss of insulin secretion) or type 2 (due to insulin resistance) diabetes (Barber, 2003). According to the International Diabetes Federation, in 2013, there were 382 million people living with diabetes and it is expected that this number will reach 471 million by 2035 (Nam Han Cho et al., 2013). As the global prevalence of diabetes increases, so will the number of people with diabetic retinopathy.

The prevalence of diabetic retinopathy increases with the duration of diabetes. In addition, hyperglycemia, hypertension and dyslipidemia are three major risk factors for developing this disease that can be controlled by patients (Yau et al., 2012).

Diabetic retinopathy is a chronic microvascular complication triggered by chronic hyperglycemia. The disease can progress from mild nonproliferative abnormalities, characterized by increased vascular permeability, to nonproliferative diabetic retinopathy that presents vascular closure, and to proliferative diabetic retinopathy, characterized by the development of fragile abnormal blood vessels along the retina and posterior surface of the vitreous (Fong et al., 2004). Total or partial vision loss can occur through vitreous hemorrhage or retinal detachment, and central vision loss can be caused by retinal leakage and subsequent macular edema (Sheetz and King, 2002).

Laser photocoagulation is an effective treatment for diabetic retinopathy. With this therapy, the areas of the retina that contain leaking blood vessels are sealed with burning laser flashes. However, many side-effects of this approach have been described. These include macular edema, impaired peripheral retinal

function and increased light sensitivity. Additionally, misplaced burns can damage retinal blood vessels (Dowler, 2003).

The exact mechanisms by which high levels of blood glucose lead to vascular disruption in diabetic retinopathy are poorly defined. However, several biochemical pathways have been proposed as potential links between hyperglycemia and diabetic retinopathy. These include increased polyol pathway, aldose reductase pathway, oxidative stress, activation of protein Kinase C pathway, accelerated formation of glycation end products (AGEs) and increased expression of growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) (Tarr et al., 2013; Zhang et al., 2011). These pathways lead to cell dysfunction and apoptosis through the activation of mitogen-activated protein kinases (MAPKs) and oxidation of intracellular components that will induce the production of angiogenic cytokines and BRB breakdown (Zhang et al., 2011).

Much of the research effort in diabetic retinopathy is often targeted at retinal vasculature, such as the administration of anti-VEGF agents (Abu El-Asrar, 2013). However, there is accumulating evidence that inflammation plays a critical role in the development of this disease (Kern, 2007).

1.4. Diabetic retinopathy and inflammation

Although microvascular changes are a hallmark of diabetic retinopathy, it has been demonstrated that the neural retina function can be compromised before the onset of vascular lesions (Antonetti et al., 2006). There is increasing evidence that inflammatory processes have a considerable role in the development of this disease. The loss of chromatic discrimination, contrast sensitivity and dark adaptation has been detected using electrophysiological methods both in diabetic patients (Frost-Larsen et al., 1980) and in diabetic animals (Matsubara et al., 2006) before microvascular alterations could be detected. In addition, loss of RGCs and glial activation has been detected in diabetic humans (Barber et al., 1998) and rats (Seki et al., 2004). All of these alterations together can be considered neurodegenerative and precede microvascular abnormalities, indicating that neural impairment occurs before the alterations in the BRB. Diabetes induces the dysregulation of the nitric oxide

synthase pathway, increases NO production, nuclear factor-kB (NF-κB) activation and the release of pro-inflammatory cytokines (TNF, IL-1β and IL-6), upregulates intracellular adhesion molecule-1 (ICAM-1) and increases leukostasis (Zeng et al., 2008; Zhang et al., 2011).

These findings offer new insights into the pathogenesis of diabetic retinopathy and the possibility to develop novel therapeutic approaches to treat this disease.

1.5. Neuropeptide Y

Neuropeptide Y (NPY) is a 36-amino-acid peptide that was first isolated in 1982 from porcine brain (Tatemoto, 1982) and was proven to be highly conserved throughout evolution (Larhammar, 1996). It is a neurotransmitter/neuromodulator synthesized by neurons and is one of the most abundant peptides in the central and peripheral nervous system (Gray and Morley, 1986). NPY belongs to the family of peptide tyrosine-tyrosine (PYY) and the pancreatic polypeptide (PP). These three peptides share considerable amino acid homology, amidated C-terminal ends, and the presence of a large number of tyrosine residues (Larhammar, 1996). They share a common tertiary structure of an alpha-helix and polyproline helix connected by a beta-turn that results in a characteristic U-shaped peptide known as a PP-fold (Berglund et al., 2003) (Fig. 6). PYY is predominantly synthesized and released by L cells in the distal ileum and colon (Adrian et al., 1987). PP is mainly found in pancreatic cells, distinct from those producing insulin, glucagon or somatostatin (Larhammar, 1996). These two peptides act as hormones and are mainly associated with the regulation of food intake (Simpson et al., 2012).

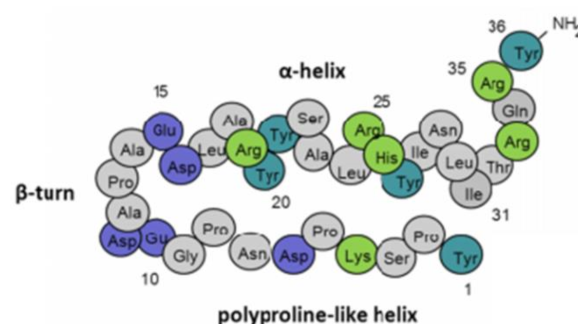


Fig. 6 – Tertiary structure of porcine NPY (Kaske, 2012).

Sympathetic neurons are the main source of NPY in the periphery, where it is co-localized with norepinephrine (NE) (Ekblad et al., 1984). In the CNS, it is one of the most abundant and widespread peptides, being associated with several physiological and pathological conditions. Among other functions, this peptide is involved in the modulation of pain, memory, eating behavior and anxiety (Wettstein et al., 1995).

1.5.1. Biosynthesis and metabolism

The expression of NPY gene results in a 97-amino-acid pre-pro-NPY (Minth et al., 1984). In order to synthesize the mature form of NPY1-36, a series of proteolytic steps takes place. A signal peptidase cleaves the signal peptide of pre-pro-NPY originating pro-NPY, and then a prohormone convertase cleaves pro-NPY at a dibasic site thus removing the C-terminal peptide of NPY (CPON) from NPY1-39 (Funkelstein et al., 2012; Funkelstein et al., 2008). NPY1-39 is further processed by carboxypeptidase into NPY1-37. Finally, a peptidylglycine α-amidating monooxygenase is responsible for the C-terminal amidation of NPY, which results in the mature and biological active form of NPY (NPY1-36) (Fig. 7). The mature NPY1-36 can be cleaved in the serum into three main fragments: NPY3-36, NPY3-35, and NPY2-36 (Abid et al., 2009). Dipeptidyl peptidase IV (DPP-IV) is a major enzyme responsible for NPY metabolism. DPPIV cleaves NPY1-36 into NPY3-36. Alternatively, NPY1-36 is processed by aminopeptidase P into NPY2-36. NPY3-36 and NPY2-36 loose the affinity for Y_1 receptor while maintaining high affinity for Y_2 and Y_5 receptors. In addition, the enzyme kallikrein can further cleave NPY3-36 into NPY3-35 which does not bind to any of NPY receptors and is likely a metabolic clearance product of NPY3-36.

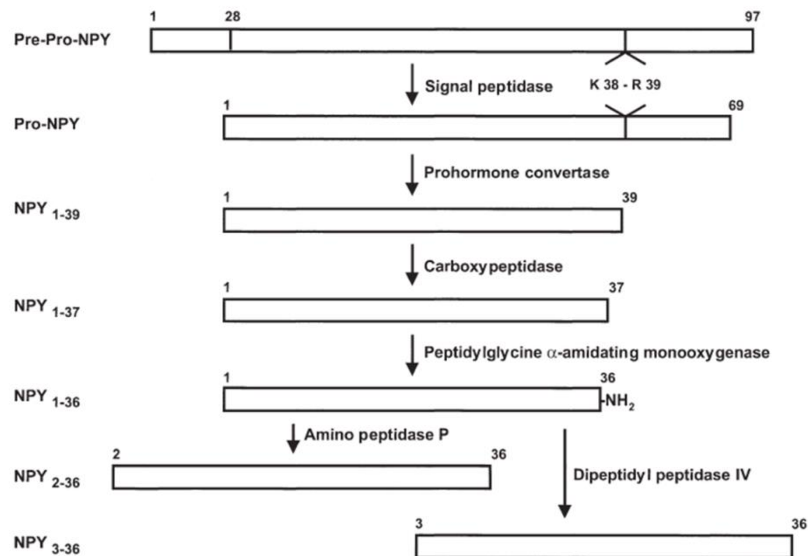


Fig. 7 - Biosynthesis and metabolization of NPY. NPY gene is composed of four exons and results in the synthesis of a 97-amino-acid pre-pro-NPY. After a series of proteolytic steps and final C-terminal amidation, the mature and biological active NPY_{1-36} is formed (Pedrazzini et al., 2003).

1.5.2. NPY Receptors

Functional active NPY receptors, Y_1, Y_2, Y_4, and Y_5, in humans and rats, are all G protein-coupled receptors, consisting of an extracellular N-terminus, seven transmembrane domains and an intracellular C-terminus. These receptors are widely distributed throughout the rat brain, especially in regions such as hypothalamus, hippocampus, and amygdala (Parker and Herzog, 1999). The Y_1 receptor binds to NPY family peptides with an order of potency of NPY ≥ PYY ≥ [Pro^{34}] substituted analog >> C-terminal fragment > PP (Michel et al., 1998). Y_1 receptor has been found in several brain regions, namely the cerebral cortex, hippocampus, hypothalamus, thalamus, amygdala, and brainstem (Shaw et al., 2003; Wolak et al., 2003). The Y_2 receptor typically binds NPY family peptides with an agonist order of potency of NPY ≈ PYY ≥ C-terminal fragment >> [Pro^{34}]substituted analog > PP (Michel et al., 1998), and it is also widely distributed in the brain, namely the thalamus, caudate nucleus, hippocampus, and cerebellum. The Y_4 receptor typically binds preferentially to PP (13.8 pM, K_i) (Lundell et al., 1995), though it also binds NPY and PYY (9.9 and 1.44 nM, respectively). Y_4 receptor is mainly found in peripheral nervous system though low levels are also found in the brain (Parker and Herzog, 1999). The Y_5

receptor binds NPY family peptides (NPY ≥ PYY ≈ [Pro^{34}]substituted analog ≈ NPY2-36 ≈ PYY3-36 >> NPY13-36) and it is also found in different brain regions, such as olfactory bulb, lateral septum, thalamus, hippocampus, amygdala, brainstem, nucleus tractus solitarius and area postrema (Dumont et al., 1998; Wolak et al., 2003). Up to date, Y_3 receptor has not been cloned and no specific agonist or antagonist has been described, and initial studies reporting Y_3 may have result from experimental artefacts (Michel et al., 1998). Regarding Y_6 receptor, first cloned from mouse genomic DNA (Weinberg et al., 1996), and although a human homologous has been cloned, it does not result in a functional protein (Gregor et al., 1996).

1.6. Neuropeptide Y system in the retina

The NPY presence in the retina has been reported in many different species, such as frog, chicken, pig, rabbit, cat, rat and mouse (Santos-Carvalho et al., 2014). In mouse and rat retina, NPY is mainly present in amacrine cells and displaced amacrine cells in GCL, co-localizing mainly with gamma-aminobutyric acid (GABA) (Oh et al., 2002; Sinclair and Nirenberg, 2001). In human retina, NPY has been reported in amacrine cells and RGCs (Jen et al., 1994; Jotwani et al., 1994). In addition, in primary retinal cell cultures NPY is found in different rat retinal cells such as neurons, endothelial cells, microglial cells and Müller cells (Alvaro et al., 2007). Moreover, Y_1 receptor is detected in horizontal and amacrine cells of rat retina (D'Angelo et al., 2002). In primary retinal neural cell cultures, Y_1 and Y_2 receptors are found in different neuronal types and glial cells (Santos-Carvalho et al., 2013). In addition, in the disease human retina, Y_1 receptor is mainly detected in glial cells (Canto Soler et al., 2002). The role of NPY in retinal physiology remains poorly studied. Nevertheless, NPY has been found to regulate neurotransmitter release (Bruun and Ehinger, 1993), possibly via regulation of calcium influx (Alvaro et al., 2009). In addition, NPY might also be involved in the regulation of RGC physiology (Sinclair et al., 2004) and Müller glial cell swelling in the rat retina (Uckermann et al., 2006).

1.7. Neuropeptide Y and microglia

Increasing evidence shows that NPY plays an important role in the immune system (Dimitrijevic and Stanojevic, 2013). This peptide has been shown to modulate several actions that include cytokine production and release, NO and ROS production and the activation of immune cells (Bedoui et al., 2007; Ferreira et al., 2011; Ferreira et al., 2010). Among other immune cells, it has been demonstrated that NPY is present in retinal microglial cells (Alvaro et al., 2007) and in N9 microglial cell line (Ferreira et al., 2010). In fact, it was demonstrated that NPY, by the activation of Y_1 receptor, inhibits microglia cell activation, the release of IL-1β and the production of NO (Ferreira et al., 2010). Additionally, the activation of Y_1 receptor by NPY also inhibited microglia cell motility and phagocytic capacity (Ferreira et al., 2012). These data suggests that NPY, through the activation of Y_1 receptor, can prevent the excessive microglial activity, avoiding the overproduction of pro-inflammatory mediators and phagocytic aggressive behavior.

1.8. Sitagliptin and DPP-IV

DPP-IV, also known as CD26, is a membrane-associated peptidase. Being widespread throughout the body, it has pleiotropic biological activities. In fact, DPP-IV has several substrates, including NPY, glucagon-like peptide-1 (GLP-1), PYY, gastric inhibitory polypeptidase, and paracrine chemokines (Mentlein, 1999).

Sitagliptin was the first DPP-IV inhibitor to be used in clinical practice. It is used for the treatment of type 2 diabetes in monotherapy or in combination with other antihyperglycemic agents, such as metformin or thiozolidinedione (Garg et al., 2013). By inhibiting DPP-IV, sitagliptin stabilizes the GLP-1 stimulating its receptor, which enhances the production of insulin (Drucker and Nauck, 2006).

Research has been made focusing the anti-inflammatory effects of DPP-IV inhibitors. Among other findings, it has been shown that the anti-inflammatory properties of NPY depend, in part, on its modulation by DPP-IV (Dimitrijevic et al., 2008). More recently, it was reported that the inhibition of DPP-IV by sitagliptin prevents the BRB breakdown, by inhibiting inflammation, apoptosis, nitrosative stress and changes in tight junctions. In addition, it was

demonstrated that these effects are independent of insulin secretion (Goncalves et al., 2012; Goncalves et al., 2014).

These evidences point DPP-IV inhibitors as potential anti-inflammatory agents, opening new perspectives for the treatment of inflammatory diseases, such as diabetic retinopathy.

Objectives

Diabetic retinopathy, a leading cause of vision loss and blindness among working-age adults, is classically considered to be a chronic retinal microvascular complication. However, increasing evidence indicate that retinal neurodegeneration is also an early event in the development of this disease. It has also been shown that low-grade inflammatory processes, associated with microglia activation, appear to have a major role in the pathogenesis of diabetic retinopathy. Sitagliptin is a DPP-IV inhibitor, which has been used since 2006 for the treatment of type 2 diabetes, by stimulating insulin secretion. Besides this effect, it has been recently reported that sitagliptin can exert anti-inflammatory effects in the retina. Moreover, DPP-IV is a key enzyme in the processing of NPY. This peptide is expressed by retinal microglial cells and there is increasing evidence indicating that NPY has anti-inflammatory properties.

Therefore, the main aims of this work were: a) to investigate the ability of sitagliptin to control retinal microglial reactivity and neuroinflammation triggered by a pro-inflammatory stimulus; b) to evaluate the potential contribution of the NPY system, and particularly the Y_1 receptor activation, for the effects of sitagliptin on microglia.

In order to achieve these goals, we used two different experimental models: primary retinal neural cell cultures and retinal organotypic cultures. Both cultures were exposed to LPS to elicit a pro-inflammatory response and were treated with sitagliptin in the absence or presence of Y_1 receptor antagonist (BIBP3226). Microglia morphology, iNOS immunoreactivity in microglial cells, iNOS expression and NO production, as well as pro-inflammatory cytokine levels (TNF and IL-1β) or immunoreactivity in microglial cells were assessed.

Chapter 2

Methods

2. Methods

2.1. Animals

All Wistar rats used in this study were kept under temperature and humidity controlled conditions, with 12h light/dark cycle and with free access to water and food. The animals were handled according with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in vision and ophthalmic research.

2.2. Primary Retinal Neural Cell Cultures

Primary retinal cell cultures were prepared from 3-5 days old rats, as previously described in (Santiago et al., 2006). The rat pups were decapitated, the eyes were enucleated and the retinas were dissected in sterile Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS; in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were then digested for 12 min, at 37ºC with 0.1% trypsin (w/v; Gibco, USA). After dissociation, cells were pelleted by centrifugation (1280 g, 1 min) and ressuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL; Life Technologies, USA) and streptomycin (100 mg/mL; Life Technologies, USA). Cells were plated at a density of $2.0x10^6$cells/cm^2 on 6-well plates or on 12-well plates with glass coverslips, pre-coated with poly-D-lysine (0.1 mg/mL; Sigma-Aldrich, USA), and maintained at 37ºC in a humidified incubator with 5% CO_2 for seven days.

2.3. Retinal Organotypic Cultures

8 to 10 weeks old wistar rats were killed by cervical dislocation. Eyes were enucleated and dissected in sterile Ca^{2+} and Mg^{2+} free HBSS and flat-mounted onto 30 mm diameter culture plate inserts with a 0.4 µm pore size (Millicell, Millipore, USA), with the GCL facing upwards. Explants were then cultured in 6-well plates containing Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) media containing GlutaMAX I (Life Technologies, USA), supplemented with heat-inactivated 10% FBS (Life Technologies, USA), 0.1% gentamicin (Life Technologies, USA). The explants were maintained for four

days *in vitro* (DIV) in a humidified incubator at 37ºC with 5% CO_2. At DIV1 and DIV2, culture medium was replaced with fresh media.

2.4. Drug Exposure

At DIV 6, cells were incubated with LPS (1 µg/mL; Sigma-Aldrich, USA) for 24h. Cell cultures were pre-treated with BIBP3226 (Y_1 receptor antagonist; 1µM; Tocris, UK), for 1 h before LPS incubation. Cells were also treated with sitagliptin (200µM; Sigma-Aldrich, USA), for 45 min before LPS incubation (Fig. 8).

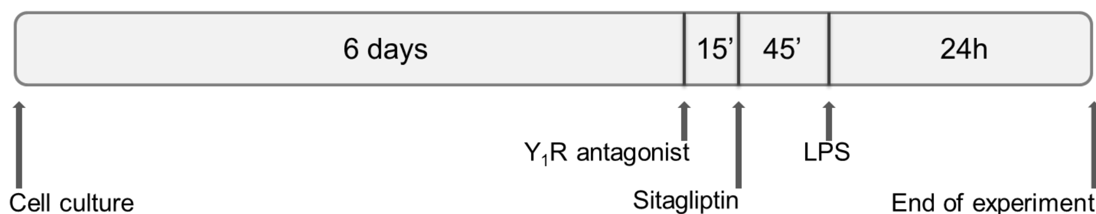
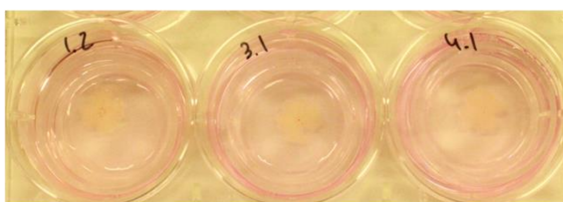


Fig. 8 - Schematic representation of drug exposure in primary retinal cell cultures.

Concerning the retinal organotypic cultures (Fig. 9A), LPS (3 µg/mL; Sigma-Aldrich, USA) was incubated at DIV3, and the retinal explants were challenged for 4h or 24h. The explants were incubated with BIBP3226 (1 µM) 1 h before LPS incubation and with sitagliptin (200µM; Sigma-Aldrich, USA), for 45 min before LPS incubation (Fig. 9B).

A



B

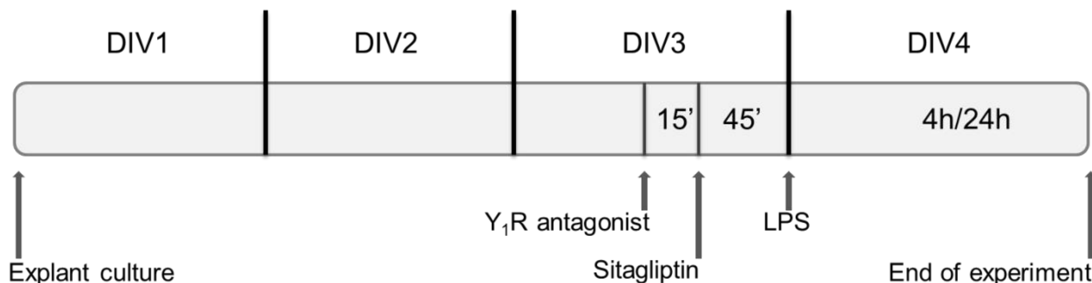


Fig. 9 – (A) Image depicting organotypic retinal cell culture. (B) Schematic representation of drug exposure in organotypic retinal cell culture.

2.5. Immunocytochemistry

Primary retinal cell cultures were washed three times with warm phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4; pH 7.4) and fixed with 4% paraformaldehyde (PFA) with 4% sucrose for 10 min at room temperature (RT). Cells were again washed three times with PBS and permeabilized with 1% Triton X-100 in PBS for 10 min at RT. Non-specific binding was prevented by incubation with 3% Bovine Serum Albumin (BSA) in PBS with 0.2% Tween-20, for 1 h at RT. Cell cultures were then incubated with primary antibodies (Table 1) in blocking solution for 90 min at RT. After being washed with blocking solution, cells were incubated with secondary antibodies (Table 1) for 1 h at RT in the dark. The cells were washed with PBS and incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:2000; Life Technologies, USA) for 10 min in the dark to stain nuclei. After washing, coverslips were mounted on glass slides with Glycergel mouting medium (Dako, Denmark). The preparations were visualized in a laser scanning confocal microscope LSM 710 (Zeiss, Germany).

2.6. Immunohistochemistry

Retinal explants were washed three times with warm PBS and fixed with ice-cold 100% ethanol for 10 min at 4ºC. Explants were again washed with PBS and non-specific binding was prevented by incubation with 3% BSA, 10% normal goat serum (NGS) and 0.1% Triton X-100, in PBS, for 1 h at RT. Samples were then incubated with primary antibodies (Table 1) in blocking solution for 48 h at 4ºC. After being washed several times with PBS, they were incubated with secondary antibodies (Table 1) overnight at 4ºC in the dark. Explants were washed with PBS and incubated with DAPI (1:1000; Life Technologies, USA) for 15 min in the dark to stain nuclei. After washing, they were flat-mounted on glass slides with the GCL facing upwards and coverslipped with Glycergel mouting medium (Dako, Denmark). The preparations were visualized in a laser scanning confocal microscope LSM 710 (Zeiss, Germany).

Table 1 - List of primary and secondary antibodies used for immunofluorescence labeling

	Host	Dilution	Supplier (catalog number)
Primary Antibodies			
Anti-CD11b	Mouse	1:100	Serotec (MCA275G)
Anti-iNOS	Rabbit	1:200	Santa Cruz Biotechnology (NOS2M-19)
Anti-IL-1β	Goat	1:100	R&D Systems (AF-501-NA)
Secondary Antibodies			
Alexa Fluor® 568 anti-mouse IgG	Goat	1:200	Invitrogen (A11004)
Alexa Fluor® 568 anti-mouse IgG	Donkey	1:200	Invitrogen (A11037)
Alexa Fluor® 488 anti-rabbit IgG	Goat	1:200	Invitrogen (A11008)
Alexa Fluor® 488 anti-goat IgG	Rabbit	1:200	Invitrogen (A11078)

2.7. Image Analysis

Stained primary cultures and explants were examined in a Zeiss LSM 710 confocal microscope (Zeiss, Germany). For each condition, 10 to 15 images were acquired randomly with a 20x objective. All images taken to compare samples were acquired using identical gain and exposure settings.

Densitometric analysis was performed for iNOS/CD11b and IL-1β/CD11b stained cells using the public domain ImageJ program (http://rsb.info.nih.gov/ij/). Each cell's immunoreactivity was calculated using the following formula: Corrected Total Cell Fluorescence = Integrated Density-(Area of selected cell x Mean Fluorescence of Background Readings), as previously described in (Gavet and Pines, 2010).

To assess differences in microglia morphology, the particle measurement feature in ImageJ was used to calculate the 2D area, perimeter, circularity and Feret's diameter of each microglia cell present in each image collected. As previously described (Kurpius et al., 2006), threshold was uniformly set to outline microglia cells and the four parameters were then automatically measured. Circularity was evaluated using the formula:

circularity=4π(area/perimeter^2). A circularity value of 1.0 indicates a perfect circular cell, and as the value approaches 0, it indicates increasingly ramified cells. Feret's diameter is a measure of the cell's length. It represents the longest distance between any two points along the selection boundary.

2.8. Nitrite Quantification Assay

Griess reaction method was used to determine NO production in retinal neural cell cultures. This is an indirect colorimetric assay that involves the spectrophotometric measurement of nitrites formed by the oxidation of NO. Cell culture medium was collected and cell debris were removed by centrifugation. Samples were then incubated (1:1) with Griess reagent mixture (1% sulfanilamide, in 5% phosphoric acid with 0.1% N-1-naphtylenediamine) for 30 min in the dark. Optical density was measured with a microplate reader (Synergy HT; Biotek, Winooski, USA) at 550 nm. Nitrite concentration was determined using a sodium nitrite standard curve.

2.9. Enzyme-Linked Immunosorbent Assay

Quantification of intracellular and extracellular pro-inflammatory cytokines – IL-1β and TNF – in primary retinal neural cell cultures was performed using enzyme-linked immunosorbent assay (ELISA).

Cells were washed with warm PBS and then they were homogenized in lysis buffer (20 mM imidazole HCl, 100 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4; pH 6.8) supplemented with protease inhibitor and kept at -80ºC for further analysis. A small aliquot was saved to measure protein concentration using the BCA protein assay kit (Pierce, USA). Cell media was collected, centrifuged and stored at -80ºC, as well.

For the assay, samples were defrosted and kept on ice and all kit's reagents were kept at RT. ELISA procedure was then performed following the instructions provided by the manufacturer (R&D Systems, USA). The absorbance was measured at 450 nm, with wavelength correction set at 540 nm, using a microplate reader (Synergy HT; Biotek, Winooski, USA). TNF and IL-1β concentrations were determined by comparison with the standard curve

using recombinant cytokines provided by manufacturer and normalized to the total amount of protein in the samples.

2.10. RNA Extraction

After washing with PBS, retinal organotypic cultures were collected and completely submerged in collection tubes containing the appropriate volume (300 μL) of RNA*later* RNA Stabilization Reagent (Qiagen, Germany) which allows tissue to be stored without jeopardizing the quality or quantity of the RNA obtained. Samples were kept at -20ºC for later processing.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany), following the manufacturer's instructions. Briefly, the samples were defrosted, and the retinal tissue was removed from RNA*later* using forceps and placed into suitably sized tubes for disruption with a lysis buffer (RLT buffer), provided with the kit. Subsequently, samples were homogenized in a QIAshredder homogenizer and transferred to RNeasy spin columns to yield an RNA enriched solution. RNA concentrations and purity ratios were then measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA).

To eliminate possible genomic DNA contamination, RNA samples were treated with Deoxyribonuclease I (DNase I, Amplification Grade, Invitrogen, Life Technologies, USA), according to manufacturer's instructions.

2.11. cDNA Synthesis

First-strand cDNA was synthesized using NZY First-Strand cDNA Synthesis Kit (Nzytech, Portugal), following supplier's instructions. NZYRT 2x Master Mix and NZYRT Enzyme Mix were added to each RNA sample followed by incubation for 10 min at 25ºC. This was followed by a 30 min at 50ºC step and the reaction was inactivated by heating at 85ºC, for 5 min. cDNA was treated with RNase H (*E. coli*) for 20 min at 37ºC to degrade the RNA template in cDNA:RNA hybrids.

cDNA synthesis and purity were evaluated using a conventional polymerase chain reaction (PCR) for β-actin using intron-spanning primers. Briefly, cDNA (1 μL) was subjected to a 35-cycle PCR amplification using 2x MyTaq Red Mix (Bioline, UK) and 200nM of forward (GCTCCTCCTGAGCGCAAG) and reverse (CATCTGCTGGAAGGTGGACA) primers. After electrophoresis on 1.5% (w/v)

agarose gel containing 0.005% (v/v) ethidium bromide, samples were visualized in a transilluminator (Versadoc, Bio-Rad, USA). cDNA was diluted 1:2 in Tris-EDTA (TE) buffer and stored at -20ºC.

2.12. Real-Time Quantitative PCR

mRNA expression of cultured retinal explants was quantified by qRT-PCR using a 2500 Real-Time PCR System (Applied Biosystems, USA).

qRT-PCR was performed using 20 µL of total reaction volume containing 10 µL of 2x iTaq^{TM}SYBR® Green Supermix (BioRad, USA), 200 nM of forward and reverse pre-optimized and costum-design primers (Table 2; Sigma-Aldrich, USA) and 2 µL of 1:2 diluted cDNA. Samples were loaded in duplicates into a 96-well plate and tested according to the following protocol:

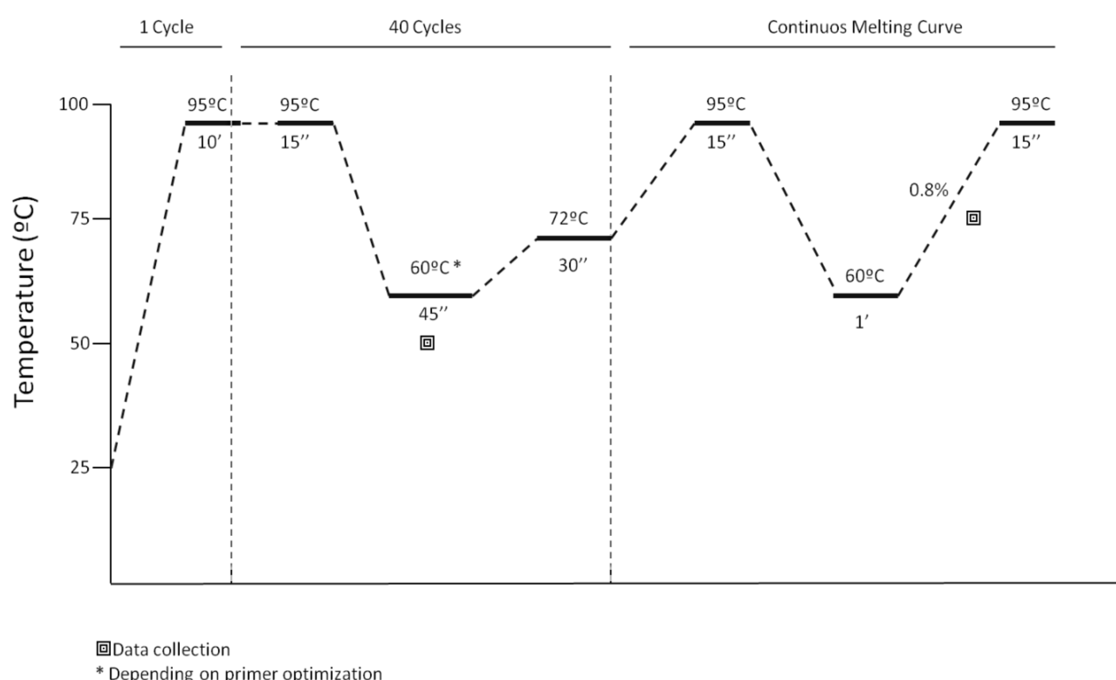


Fig. 10 - Schematic representation of qRT-PCR protocol.

Nontemplate controls were also performed in duplicate for each gene.

2.13. qRT-PCR data analysis

The expression stability of three candidate reference genes – YWHAZ, HPRT and TBP - was tested. NormFinder software (Andersen et al., 2004) identified YWHAZ as the most stable and therefore, it was used as the housekeeping gene for accurate normalization of gene expression.

Relative quantification of target gene expression was calculated using the comparative cycle threshold (Ct) method, also referred as the ΔΔCt Method. Ct values were determined for each sample and subsequently, the ΔCt was calculated using the equation: $\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$. Afterwards, ΔΔCt was determined using the following formula: $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{housekeeping\ gene})_{treated\ group} - (Ct_{target\ gene} - Ct_{housekeeping\ gene})_{control\ group}$. The relative quantification describes the change in expression of the target gene in treated samples relative to its expression in control samples. Furthermore, a melting curve analysis was performed to evaluate unspecific products and primer-dimer formation.

Table 2 - Primers used for qRT-PCR.

Genes	Forward Primer (5'-3')	Reverse Primer (3'-5')	Annealing Temperature (ºC)
Reference Genes			
Hprt	ATGGGAGGCCATCACATTGT	ATGTAATCCAGCAGGTCAGCAA	60
Tbp	ACCAGAACAACAGCCTTCCACCTT	TGGAGTAAGCCCTGTGCCGTAAG	58
Ywhaz	CAAGCATACCAAGAAGCATTTGA	GGGCCAGACCCAGTCTGA	60
Target Genes			
iNOS	AGAGACAGAAGTGCGATC	AGATTCAGTAGTCCACAATAGTA	60

2.14. Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism Version 5.00 for Windows (http://www.graphpad.com; GraphPad Software, USA). All results were submitted to non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. Differences were considered statistically significant for $p<0.05$.

Chapter 3

Results

3. Results

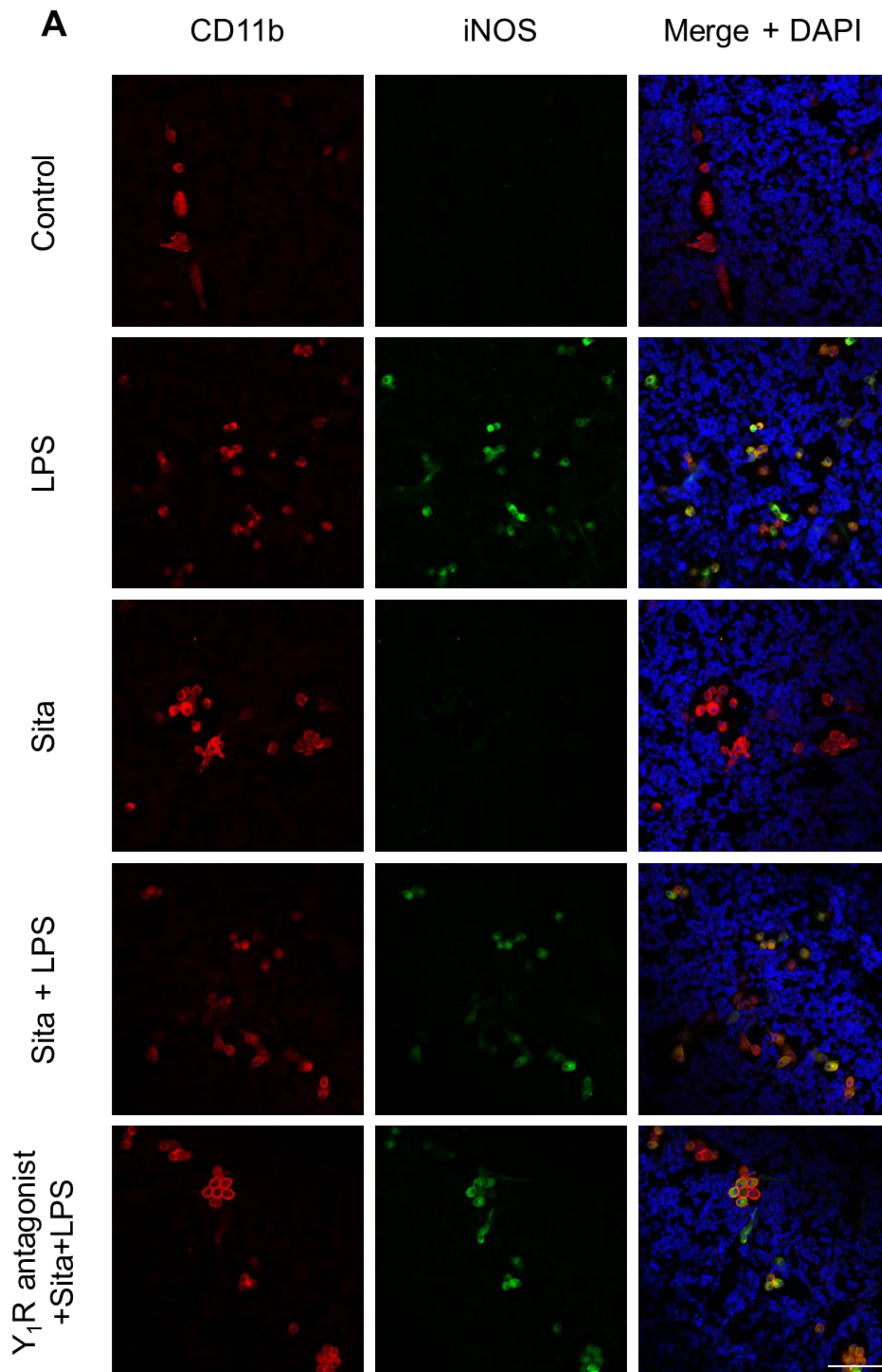
In the present work, we aimed to investigate if sitagliptin could inhibit pro-inflammatory processes in the retina, namely microglial cell activation. Moreover, we intended to investigate whether the potential inhibition of microglia activation by sitagliptin could be mediated by the NPY system, and particularly by Y_1 receptor activation. For this purpose, primary retinal neural cell cultures and cultured retinal explants were exposed to a pro-inflammatory stimulus, LPS, and the effect of sitagliptin, in the absence or in the presence of the Y_1 receptor antagonist on inflammation was evaluated.

3.1. Sitagliptin reduces LPS-triggered increase in iNOS immunoreactivity and NO production in microglia in primary retinal neural cell cultures

Inducible Nitric Oxide Synthase (iNOS) is one of the enzymes responsible for the production of NO and is typically expressed in response to cellular stress (Morris and Billiar, 1994). It has been shown that NO derived from iNOS plays a role in causing tissue damage and inflammation. Moreover, iNOS is strongly involved in the induction of early vascular changes in diabetes, suggesting that this enzyme has a key role in early diabetic retinopathy (Leal et al., 2007; Morris and Billiar, 1994).

We evaluated the modulation of LPS-induced microglia activation by sitagliptin, assessing iNOS-IR, specifically in microglia, and NO release, indirectly, by measuring nitrites in the medium (Griess reaction method). The immunoreactivity of iNOS was assessed in microglial cells after 24 h exposure to LPS (Fig. 11). Microglial cells were identified by CD11b staining. In control and sitagliptin-treated cultures (104 ± 21.7% of the control), iNOS-IR in microglial cells was barely detected. On the contrary, when cultures were incubated with 1 µg/mL LPS, iNOS-IR significantly increased in CD11b-positive cells to 1011 ± 117% of the control (Fig. 11A and 11B). When cells were treated with sitagliptin before LPS exposure, iNOS-IR in CD11b-positive cells significantly decreased to 368.1 ± 30% of the control (Fig. 11A and 11B). Incubation with Y_1 receptor antagonist (1 µM BIBP3226) before treatment with sitagliptin and exposure to LPS seemed to partly inhibit the effect of sitagliptin

(533.7 ±56.6% of the control). However, no statistically significant differences were detected.



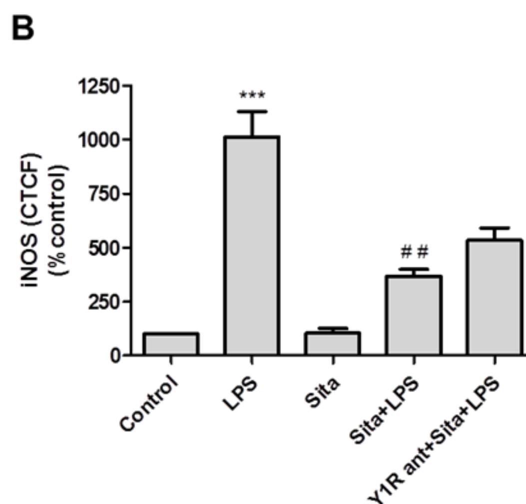


Fig. 11 - Effect of sitagliptin on the LPS-triggered increase of iNOS-IR in microglial cells in primary retinal neural cell cultures. iNOS expression was evaluated in microglia (CD11b-immunoreactive cells) in primary retinal cultures. Cells were cultured for 7 days and were exposed to LPS (1 µg/mL) for 24h in the absence or presence of 200 µM sitagliptin and with 1 µM Y_1R antagonist (BIBP3226). (A) Immunocytochemistry was performed using anti-CD11b (red) and anti-iNOS (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar: 100 µm. (B) iNOS immunoreactivity was quantified in CD11b-immunoreactive cells, in 10 random fields (20x magnification), using corrected total cell fluorescence (CTCF) formula. Data are presented as mean ± SEM of 5 independent experiments, as a percentage of control. ***p<0.001, compared to control; ##p<0.01, compared to LPS; Kruskal-Wallis, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

Additionally, the production of NO in primary retinal neural cell cultures was assessed by quantifying nitrite concentration in the culture media by Griess reaction method. In control conditions, nitrite concentration was 1.08 ± 0.18 µM, and treatment with sitagliptin alone did not induce any change (0.99 ± 0.19 µM). NO release was significantly increased after LPS exposure to 5.76 ± 0.56 µM. Pre-treatment with sitagliptin before LPS incubation significantly inhibited the increase in nitrites concentration induced by LPS. Furthermore, the effect of sitagliptin was completely abolished when cells were pre-treated with the Y_1 receptor antagonist, BIBP3226 (7.96 ± 1.3 µM), indicating an important contribution of Y_1 receptor activation mediating sitagliptin effect (Fig. 12).

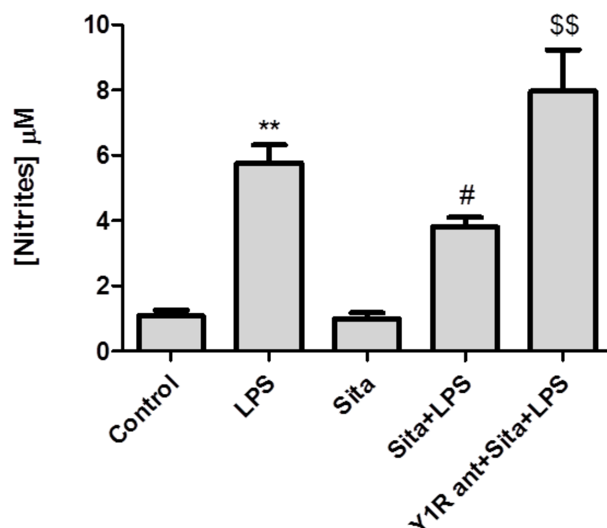


Fig. 12 - Sitagliptin partially inhibits LPS-induced increase in nitrites production and blockade of NPY Y_1 receptor abolishes this effect. The concentration of nitrites in primary retinal cell cultures media was determined by the Griess reaction method. Cells were cultured for 7 days and were exposed to LPS (1 µg/mL) for 24h in the absence or presence of 200 µM sitagliptin and with 1 µM BIBP3226 (Y_1R antagonist). Results represent the nitrites concentration (µM) and are expressed as mean ± SEM of 5 to 7 independent experiments, performed in duplicate. **p<0.001, compared to control; #p<0.05, compared to LPS; $$p<0.001, compared to Sita+LPS, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

3.2. Effect of sitagliptin and Y_1 receptor blockade in LPS-induced increase in cytokine production in primary retinal neural cell cultures

Microglia respond to proinflammatory factors becoming activated and shifting towards their cytotoxic phenotype, potentiating the inflammatory response, as they produce and release proinflammatory mediators, such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF). The release of these cytokines has been used as a marker of microglia activation.

The levels of TNF (Fig. 13) and IL1β (Fig. 14) were quantified by ELISA in primary retinal neural cell cultures, both in cell lysates (intracellular levels) and in cell culture media supernatants (extracellular levels). Exposure to 1 µg/mL LPS for 24 h increased the production and release of TNF. Quantification of TNF levels in cell lysates revealed a clear increase to 106.8 ± 26 pg/mg of protein compared to 10.5 ± 3.2 pg/mg of protein detected in control (Fig. 13A). Similarly, the extracellular TNF levels were increased to 380.6 ± 25.7 pg/mg of protein upon LPS application, compared to control (2.6 ± 0.6 pg/mg; Fig. 13B). However, pre-treatment with sitagliptin, or sitagliptin and BIBP3226 (Y_1 receptor antagonist), did not prevent the alterations triggered by LPS on either

intracellular (Fig. 13A), and extracellular TNF levels (Fig. 13B). Sitagliptin alone did not affect the TNF levels.

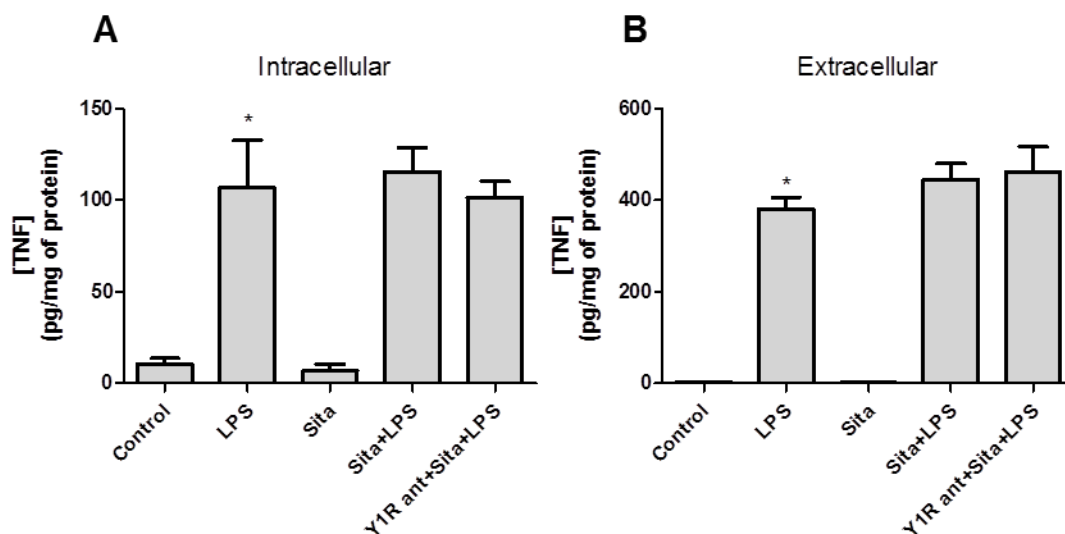


Fig. 13 - Sitagliptin does not prevent the increase of TNF levels triggered by exposure to LPS in retinal cell cultures. Primary retinal cell cultures were challenged with 1 μg/mL LPS for 24 h, in the absence or presence of 200 μM sitagliptin and 1 μM BIBP3226 (Y_1R antagonist). Intracellular (A) and extracellular (B) levels of TNF were quantified by ELISA and normalized to the total amount of protein in the samples. Results represent TNF concentration (pg/mg of protein) and are expressed as mean ± SEM of 4 independent experiments for the cell lysates and 5 to 7 for cell supernatants, both performed in duplicate. *$p<0.05$, compared to control, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

Exposure to LPS also triggered an increase in the production and release of IL-1β in primary retinal cell cultures (Fig. 14). Intracellular IL-1β levels increased from 60 ± 8.6 pg/mg of protein, in control conditions, to 9103 ± 2126 pg/mg of protein upon exposure to LPS for 24 h (Fig. 14A). Similarly, as for TNF levels, the pre-treatment with sitagliptin did not prevent the effect of LPS on intracellular IL-1β levels. Although very low amounts of IL-1β have been detected in the extracellular media in control conditions (18.81 ± 0.5 pg/mg of protein), LPS also triggered an increase in extracellular IL-1β levels to 35.7 ± 4.9 pg/mg of protein (Fig.14B). In sitagliptin-treated conditions, there was a small reduction in the extracellular IL-1β levels to 26.6 ± 1.9 pg/mg of protein, although no statistically significant differences were detected compared to LPS. Sitagliptin alone did not affect the IL-1β levels and the pre-treatment with Y_1 receptor antagonist, BIBP3226, did not also affect the levels of IL-1β in cells exposed to to LPS in the presence of sitagliptin.

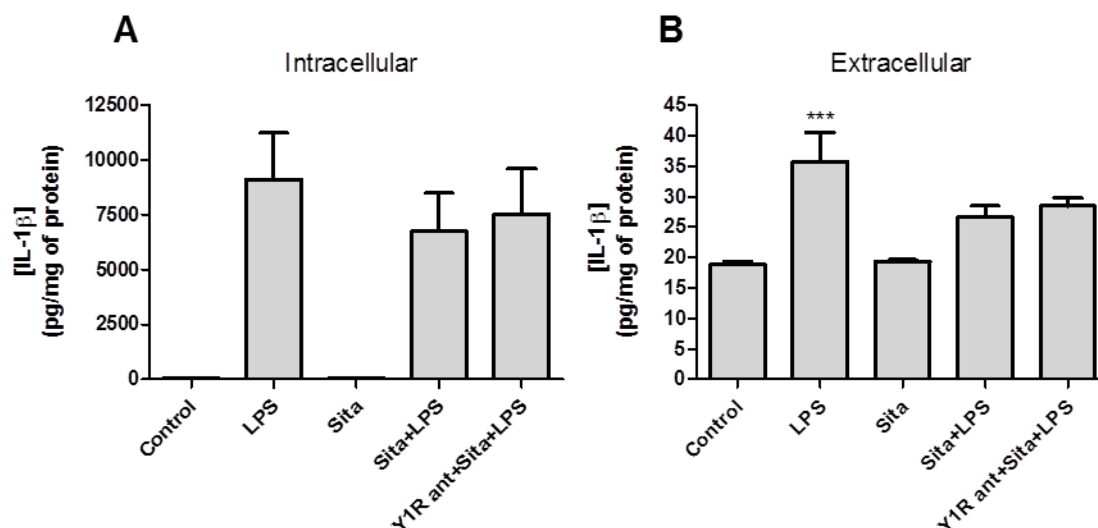
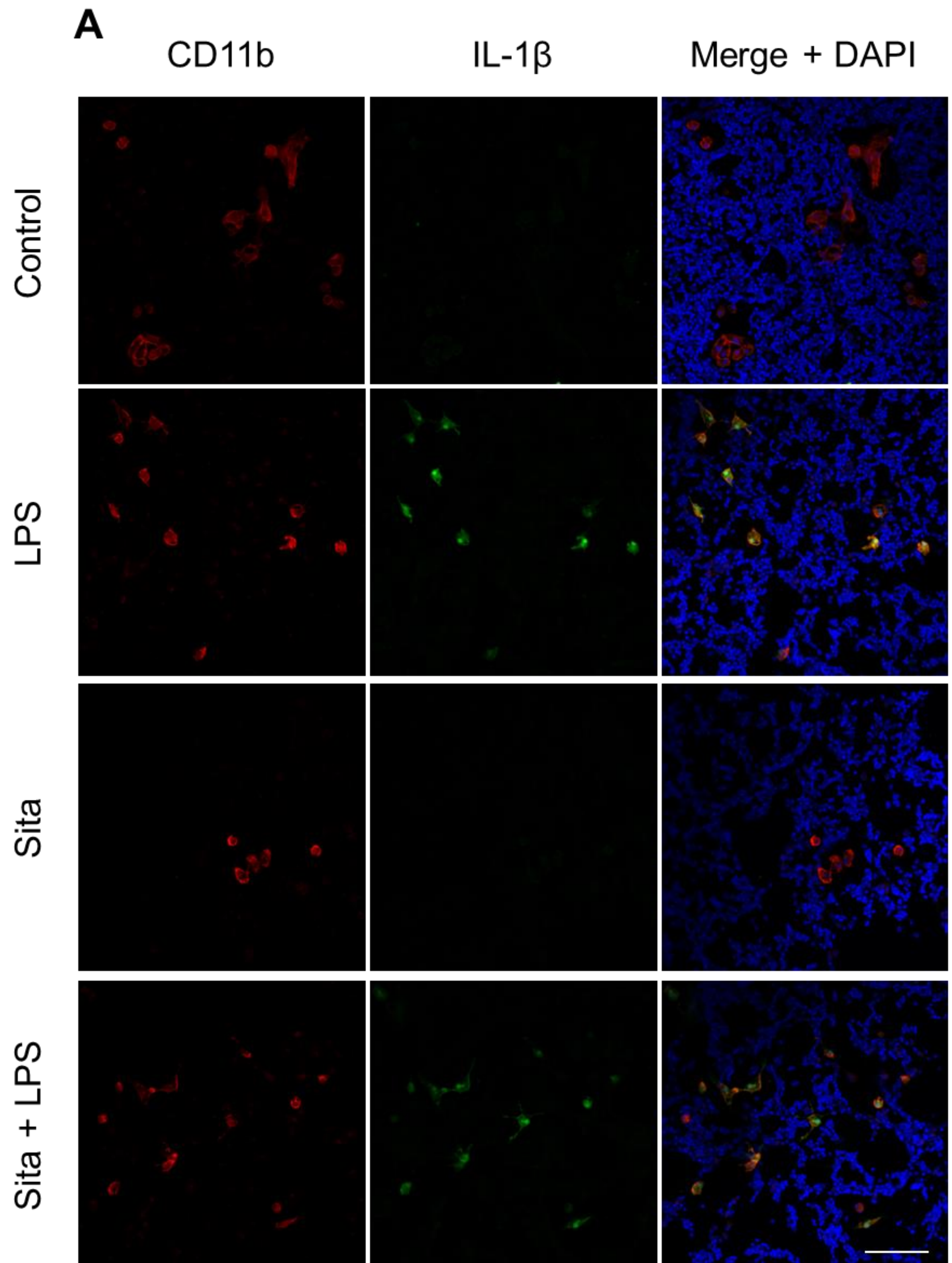


Fig. 14 - Sitagliptin does not significantly prevent the LPS-induced increase in IL-1β levels in retinal cell cultures. Primary retinal cell cultures were challenged with 1 μg/mL LPS for 24h, in the absence or presence of 200 μM sitagliptin and 1 μM BIBP3226 (Y_1R antagonist). Intracellular (A) and extracellular (B) levels of IL-1β were quantified by ELISA and normalized to the total amount of protein in the samples. Results represent IL-1β levels (pg/mg of protein) and are expressed as mean ± SEM of 4 independent experiments for the cell lysates and 5 to 7 for cell supernatants, both performed in duplicate. ***$p<0.001$, compared to control, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

IL-1β immunoreactivity (IL-1β-IR) was also assessed in primary retinal neural cell cultures. We quantified IL-1β-IR in microglial cells, labeled with an anti-CD11b antibody. In both controls and cultures treated with sitagliptin conditions, a very faint staining was detected for IL-1β (Fig. 15A). However, when cells were stimulated with LPS, IL-1β-IR in microglial cells significantly increased to 1138 ± 8% of the control (Fig. 15A and 15B). Pre-treatment with sitagliptin appears to prevent the LPS-induced IL-1β-IR increase in microglial cells (535.3 ± 40% of the control, Fig. 15A and 15B). However, these are still preliminary results, with a small number of independent experiments (n=2) and thus statistical significance was not assessed.


A
CD11b
IL-1β
Merge + DAPI
Control
LPS
Sita
Sita + LPS

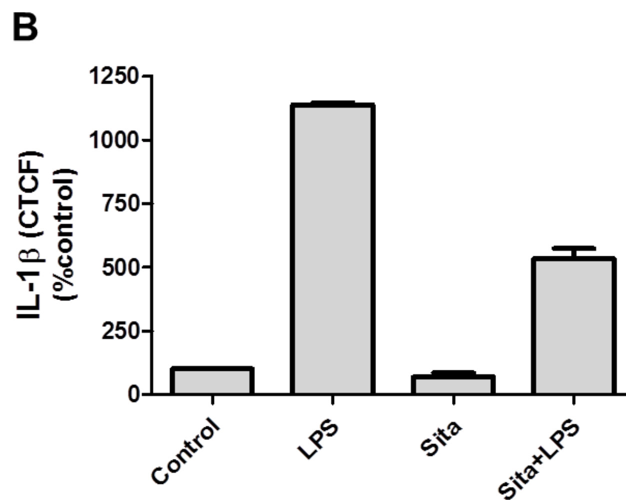


Fig. 15 - Sitagliptin appears to inhibit LPS-induced increase in IL-1β immunoreactivity (IL-1β-IR) in microglial cells. IL-1β-IR was evaluated in microglia in primary retinal cell cultures. Cells were cultured for 7 days and were exposed to LPS (1 μg/mL) for 24h in the absence of presence of 200 μM sitagliptin. (A) Immunocytochemistry was performed using anti-CD11b (red) and anti-IL-1β (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar: 100 μm. (B) iNOS-IR was quantified in CD11b-IR cells, in 10 random fields (20x magnification), using corrected cell fluorescence (CTCF) formula. Data are presented as mean ± SEM of 2 independent experiments, as a percentage of control. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

3.3. Sitagliptin prevents LPS-induced alterations in microglia morphology in cultured retinal explants

Under physiological conditions microglial cells present a ramified morphology, which is associated with a surveillance phenotype. Changes in homeostasis may lead microglia to adopt a less ramified morphology, in agreement with a reactive phenotype (Kettenmann et al., 2011). Therefore, we investigated whether sitagliptin could modulate microglia reactivity elicited by LPS in cultured retinal explants, by assessing morphological alterations after CD11b labelling. Using confocal microscopy, we acquired images from microglial cells at the level of GCL, where there is a high density of microglial cells in the retina. We also evaluated whether the potential effect of sitagliptin could be mediated by the involvement of the NPY Y_1 receptor. Cultured retinal explants were exposed to LPS (3 μg/mL) for 24 h to trigger an inflammatory response. In control conditions microglial cells are typically ramified, and upon LPS stimulation they present an amoeboid-like morphology with fewer ramifications (Fig. 16A). In order to evaluate the changes in microglia morphology, we assessed four different morphological parameters in microglial cells: area, perimeter, circularity and Feret's diameter. Exposure to 3 μg/mL LPS for 24 h resulted in a significant

decrease in cell area from 873 ± 101.1 μm^2 in control conditions to 492.1 ± 25.7 μm^2 (Fig.16A and 16B). Pre-treatment with sitagliptin prevented the decrease in the area of microglial cells triggered by LPS (1017 ± 81.8 μm^2) (Fig. 16A and 16B). Upon LPS stimulation, cell perimeter also diminished to 148.4 ± 5.3 μm comparing to the cell area in control conditions (241.8 ± 15.8 μm) and this effect was prevented by sitagliptin (257 ± 6.4 μm) (Fig. 16A and 16C). Feret's diameter also decreased from 62.23 ± 3.4 μm in control conditions to 39.9 ± 0.9 μm after LPS exposure and, similarly to the above mentioned parameters, sitagliptin inhibited this decrease (61.3 ± 2.1 μm) (Fig. 16A and 16D). On the other hand, the circularity index of microglial cells was significantly higher in LPS-treated explants (0.33 ± 0.1 μm), when compared to control (0.20 ± 0.01 μm) (Fig. 16A and 16E). Pre-treatment with sitagliptin prevented the increase in circularity index of microglia (0.22 ± 0.01), when compared to LPS condition (Fig. 16A and 16E). Sitagliptin alone did not affect any of the analyzed parameters. Pre-treatment with the Y_1 receptor antagonist (1 μM BIBP3226) appears to slightly inhibit the effects of sitagliptin pre-treatment on the parameters analyzed, pointing to a potential partial involvement of the Y_1 receptor on the effects of sitagliptin. However, no statistically significant differences were observed, and so, altogether, these results suggest that the anti-inflammatory effect of sitagliptin against microglia activation appears to be independent of NPY Y_1 receptor activation.

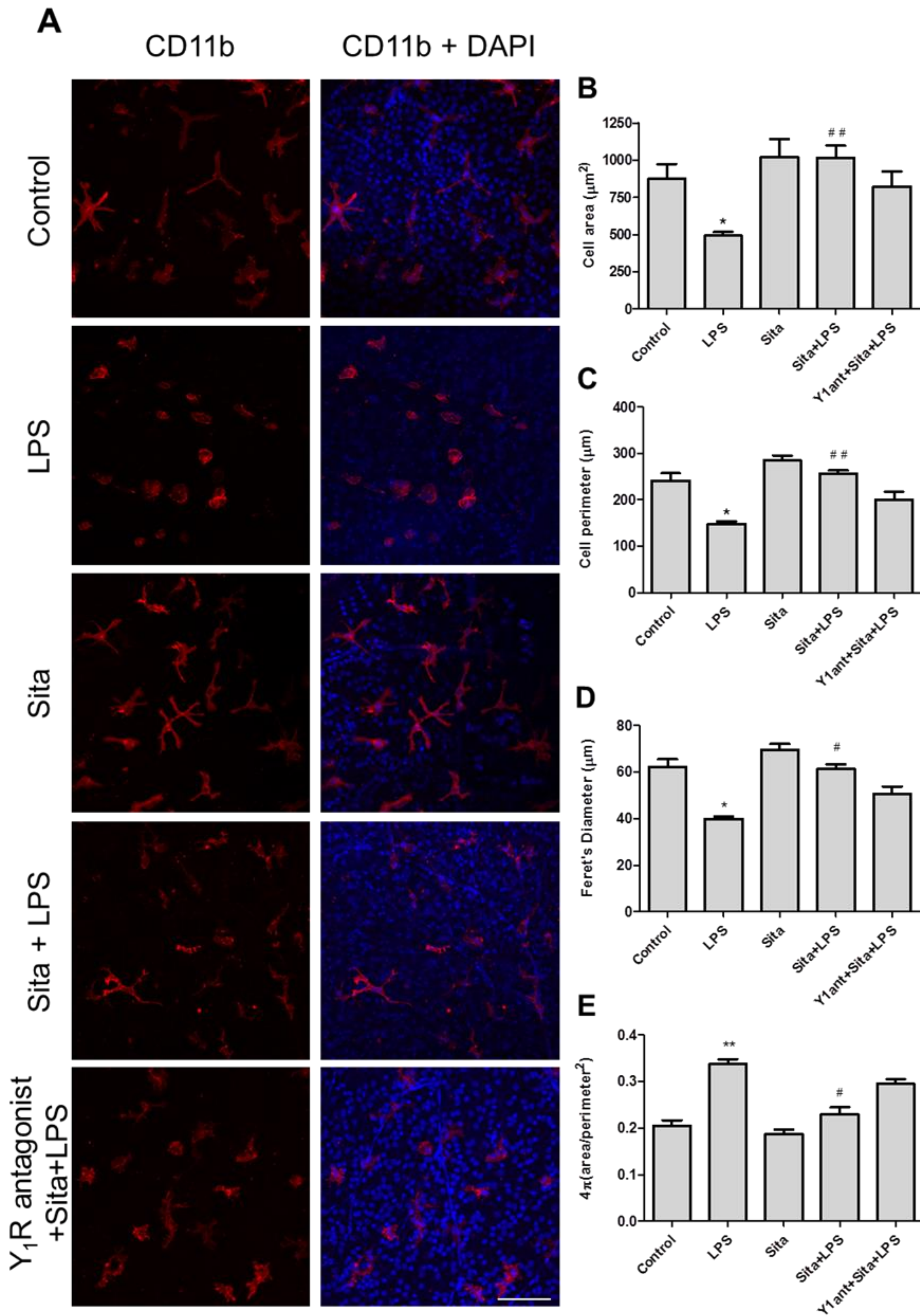


Fig. 16 - Sitagliptin inhibits morphological changes in retinal microglia triggered by LPS. The analysis of microglia morphology was performed in cultured retinal explants. Explants were cultured for 4 days and were exposed to LPS (3 µg/mL), at day 3 in culture, for 24h, in the absence or presence of 200 µM sitagliptin and 1 µM BIBP3226 (Y_1R antagonist). (A) Immunohistochemistry was performed using an anti-CD11b (red) antibody. Nuclei were counterstained with DAPI (blue). Scale bar: 100 µm. Microglia area (B), perimeter (C), Feret's

diameter (D) and circularity (E) were evaluated in CD11b-IR cells. In each explant, 12 random fields (3 fields per retinal quadrant) were analyzed (20x magnification). Data are presented as mean ± SEM of 4-5 independent experiments. *p<0.05, compared to control; **p<0.01, compared to control; #p<0.05, compared to LPS; ##p<0.01, compared to LPS, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

3.4. Sitagliptin reduces the increase in iNOS immunoreactivity in microglial cells triggered by LPS in cultured retinal explants

In order to confirm the results obtained in primary retinal neural cell cultures (Fig.11), the effect of sitagliptin on iNOS-IR upon LPS stimulation was assessed in cultured retinal explants. Firstly, we analyzed the iNOS mRNA levels, which were normalized to YMHAZ. Exposure to 3 μg/mL LPS for 4 h significantly increased iNOS mRNA expression by 121.9 ± 8.9 fold, when compared with control (Fig. 17). Treatment with sitagliptin did not prevent this increase (110.1 ± 16.3 fold increase). However, pre-treatment with the Y_1 receptor antagonist (1 μM BIBP3226) resulted in significantly higher levels of iNOS mRNA expression (189.2 ± 23.24 fold increase), when compared to LPS (Fig. 17). This result suggests that basal activation of Y_1 receptor by endogenous NPY might regulate iNOS expression. Sitagliptin alone did not affect the iNOS mRNA levels.

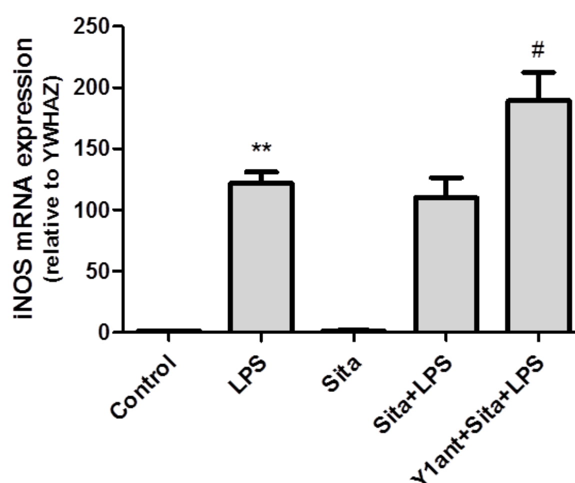
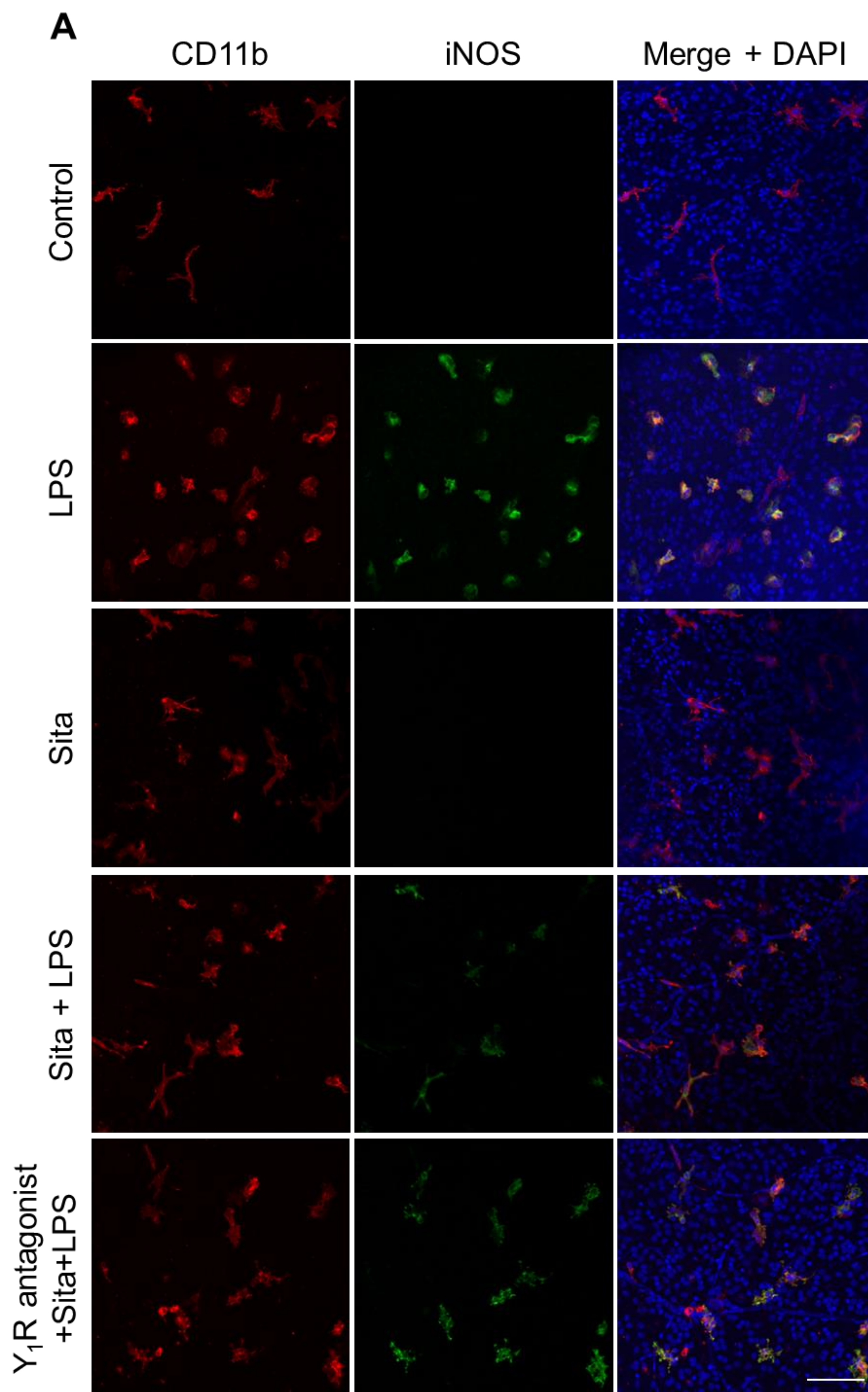


Fig. 17 - Sitagliptin does not affect the increase in iNOS mRNA expression triggered by LPS in cultured retinal explants, but treatment with Y_1 receptor antagonist exacerbates LPS effect. After 3 days in culture, retinal explants were challenged with 3 μg/mL LPS for 4h, in the absence or presence of 200 μM sitagliptin and 1 μM BIBP3226 (Y_1R antagonist). iNOS mRNA expression in cultured retinal explants was assessed by qRT-PCR. Data are presented as mean ± SEM of 6 independent experiments. **p<0.01, compared to control; #p<0.05,

compared to LPS, Kruskal-Wallis test, followed by Dunn's multiple comparison test. Sita: sitagliptin; Y_1R ant: Y_1R antagonist.

Moreover, iNOS-IR was also quantified in microglia in cultured retinal explants. Microglia were identified by CD11b immunostaining and analyzed in the GCL. Sitagliptin per se did not affect the iNOS-IR in microglial cells. Upon 24 h exposure to LPS, iNOS-IR significantly increased (2960 ± 289.3% of the control) in microglial cells (Fig. 18A and 18B). When explants were treated with sitagliptin before exposure to LPS, iNOS-IR was significantly inhibited (1535 ±137.9% of the control) when compared to LPS condition (Fig. 18A and 18B). Pre-treatment with the Y_1 receptor antagonist (1 μM BIBP3226) did not affect the effect of sitagliptin.


A
CD11b
iNOS
Merge + DAPI
Control
LPS
Sita
Sita + LPS
Y_1R antagonist
+Sita+LPS

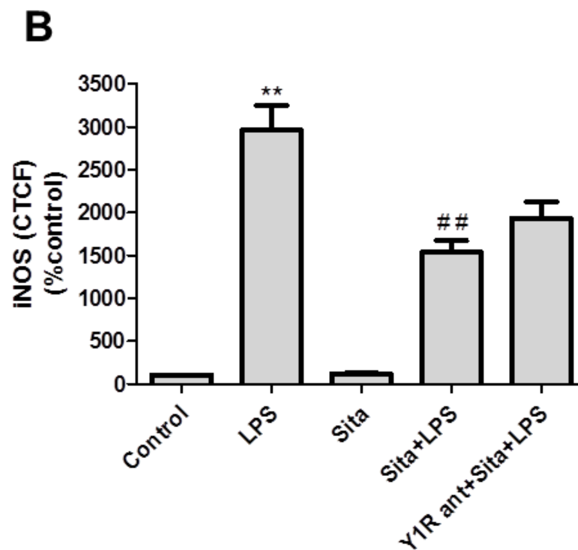


Fig. 118 - Sitagliptin reduces the increase in iNOS-IR triggered by LPS in microglial cells in cultured retinal explants. iNOS expression was evaluated in microglia localized in the GCL in cultured retinal explants. Explants were cultured for 4 days and exposed at day 3 in culture to LPS (3 µg/mL) for 24h, in the absence or presence of 200 µM sitagliptin and 1 µM BIBP3226 (Y_1R antagonist). (A) Immunohistochemistry was performed using anti-CD11b (red) and anti-iNOS (green) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar: 100 µm. (B) iNOS immunoreactivity was quantified in CD11b-IR cells. In each explant, 12 random fields (3 fields per retinal quadrant) were analyzed (20x magnification), using corrected cell fluorescence (CTCF) formula. Data are presented as mean ± SEM of 6 independent experiments, as a percentage of control. **p<0.01, compared to control; ##p<0.01, compared to LPS, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

Chapter 4

Discussion

4. Discussion

Sitagliptin is a DPP-IV inhibitor that enhances insulin secretion and is used in the treatment of type 2 diabetes. Sitagliptin treatment was shown to prevent the breakdown of the BRB and the increase of IL-1β immunoreactivity in the retinas of type 1 and 2 diabetic animals (Goncalves et al., 2012; Goncalves et al., 2014). However, this effect was not due to the normalization of glucose levels or insulin secretion, thus suggesting that sitagliptin can have anti-inflammatory effects in the retina. Taking these evidences into account, we hypothesized that sitagliptin is able to inhibit retinal microglia reactivity.

DPP-IV cleaves NPY1-36 to NPY3-36, and although NPY1-36 binds to Y_1 and Y_2 receptors with similar affinity, NP3-36 is an Y_2/Y_5 receptor preferring peptide (Grandt et al., 1996; Mentlein, 1999). Therefore, DPP-IV is a key enzyme in the regulation of NPY as its activity terminates NPY action on Y_1 receptor. Since NPY can have anti-inflammatory effects on microglial cell cultures via Y_1 receptors (Ferreira et al., 2010), we also hypothesized that the effects of sitagliptin could be mediated through the activation of Y_1 receptor by NPY. In addition, preliminary results obtained in our lab, in cultured retinal explants, showed that NPY and Y_1 receptor agonist inhibited LPS-induced microglial reactivity and the increase in iNOS expression in microglial cells localized in the GCL.

To test these hypothesis, we used primary retinal neural cell cultures and cultured retinal explants that were pre-incubated with sitagliptin and with BIBP3226, an antagonist of the Y_1 receptor. Because inflammation plays a critical role in diabetic retinopathy (Kern, 2007), we exposed both cultures to LPS, to trigger a pro-inflammatory response, in order to test the anti-inflammatory effects of sitagliptin on microglia. LPS is a major component of the outer membrane of Gram-negative bacteria that binds to toll-like receptors 4 (TLR4), expressed by microglia, leading to the activation of several signaling cascades and secretion of pro-inflammatory cytokines (Lehnardt et al., 2003).

It has been reported that changes in microglia morphology are associated with alterations in microglia reactivity. In healthy conditions, microglial cells are highly ramified. Upon an insult, microglial cells undergo a transformation from resting to activated state and become amoeboid (Kettenmann et al., 2011). In

the present study, we evaluated four different morphological parameters (area, perimeter, Feret's diameter and circularity) in microglial cells (CD11b-immunoreactive cells) in retinal organotypic cultures. Upon LPS stimulation, we observed that microglial cells presented an amoeboid-like morphology with fewer ramifications. Pre-treatment with sitagliptin resulted in a rescue of the ramified phenotype, indicating that sitagliptin has an anti-inflammatory effect, exerted directly on the immune cells of the CNS, which may account for the anti-inflammatory effects of sitagliptin detected in the retina of diabetic animals (Goncalves et al., 2012; Goncalves et al., 2014). In all the four parameters measured, there was a small inhibition, although not significant, of the sitagliptin effect when Y_1 receptor was blocked, thus suggesting that the inhibitory effect of sitagliptin on microglia activation appears not be mediated by the NPY system, but rather through other mechanisms.

Early microglia activation in the retina is known to be induced by ischemia, autoimmune mechanisms, neuronal injury, ocular infections, and metabolic and hereditary retinopathies (Schuetz and Thanos, 2004). When microglial cells become activated, they can release several molecules, which can be neurotoxic, depending on their concentration, such as NO. It has been described that NO derived from iNOS is strongly involved in the induction of early vascular changes, suggesting that this enzyme has a key role in early diabetic retinopathy (Leal et al., 2007). In this work, exposure to LPS increased iNOS-IR in microglial cells present both in retinal primary neural cell cultures and in retinal organotypic cultures. Although other cells present in these cultures (astrocytes, Müller cells, and neurons) are known to express iNOS (Saha and Pahan, 2006; Wang et al., 2011), in this study, iNOS-IR was observed almost exclusively in microglial cells. This result is consistent with several studies that show that microglial cells produce high amounts of NO by iNOS, after stimulation with LPS (Fiebich et al., 1998; Lieb et al., 2003; Zhang et al., 2012). Pre-treatment with sitagliptin significantly inhibited the increase in iNOS-IR induced by LPS, thus confirming the anti-inflammatory effects of sitagliptin.

Both in primary retinal neural cell cultures and in retinal organotypic cultures, incubation with Y_1 receptor antagonist seemed to partly inhibit the effect of sitagliptin in iNOS-IR, but the effect was not statistically significant. One

possible explanation for this observation is that NPY1-36 can be activating not only Y_1 receptors but Y_2 receptors as well, which are also present in retinal microglial cells (Santos-Carvalho et al., 2013) and have been described to be overexpressed by immune cells upon LPS insult (Nave et al., 2004). In order to test this, additional experiments, for example blocking the Y_2 receptor, need to be performed. Besides this possibility, there are also other pathways being affected by DPP-IV inhibition. In fact, sitagliptin is used for the treatment of type 2 diabetes because the inhibition of DPP-IV increases the intact levels of GLP-1 (Drucker and Nauck, 2006). It has recently been described that exendin-4, a GLP-1 agonist, is able to inhibit LPS-induced iNOS expression and nitrite production in Raw264.7 macrophage cells (Chang et al., 2013).

Despite the inhibitory effect of sitagliptin on iNOS-IR in microglial cells exposed to LPS, in cultured retinal explants sitagliptin was not able to prevent the increase in iNOS mRNA expression. In fact, iNOS mRNA expression and iNOS-IR were evaluated at different time points after exposure to LPS (4 h for iNOS mRNA expression and 24 h for iNOS-IR) and this fact might explain the discrepancy in the results. However, for many proteins in different biological systems it is not uncommon getting divergent results when mRNA and protein expression are compared. Moreover, the blockade of Y_1 receptor exacerbated the effect of LPS in iNOS mRNA expression, suggesting that basal activation of Y_1 receptor by endogenous NPY might have a role in regulating iNOS expression in retinal tissue.

In accordance with the results obtained for iNOS-IR and expression, LPS increased the levels of NO released by primary retinal neural cell cultures. Production and release of NO by N9 murine microglial cells after LPS has been previously reported (Dimayuga et al., 2007; Ferreira et al., 2010). Pre-treatment with sitagliptin before LPS incubation partially inhibited the increase in the concentration of nitrites triggered by LPS. Furthermore, the Y_1 receptor antagonist completely abolished the effect of sitagliptin, indicating an important contribution of the activation of the Y_1 receptor by NPY for the protective effect of sitagliptin against the increase in NO levels. In line with these findings, the inhibition of LPS-induced NO production by NPY has been reported in murine N9 microglia cell line and it was demonstrated that this effect involved the activation of Y_1 receptor (Ferreira et al., 2010). Regarding the results of the

effect of sitagliptin, and the Y_1 receptor antagonist, on iNOS mRNA expression, iNOS-IR, and NO production (Griess method) either in primary retinal neural cells cultures or cultured retinal explants, there is apparently some discrepancy, particularly related with the effect of the the Y_1 receptor antagonist. In fact, BIBP3226 antagonized the effects of sitagliptin when the NO levels were indirectly measured by the Griess reaction, but the same antagonist was not able to significantly affect the inhibitory effect of sitagliptin on iNOS-IR. However, we must keep in mind that we are dealing with different techniques that are measuring different parameters and have different sensitivities. Moreover, it is important to emphasize that there was a clear tendency (not statistically significant), for a partial antagonizing effect of the Y_1 receptor antagonist against the effect of sitagliptin on iNOS-IR, suggesting that we cannot completely exclude the involvement of the NPY system, and particularly the Y_1 receptor, on the effects of sitagliptin. In fact, the involvement of the Y_1 receptor was clearly seen in the experiments using the Griess reaction.

To further test the potential inhibitory effect of sitagliptin on retinal microglia activation and neuroinflammation, its effect on the production of cytokines, namely TNF and IL-1β, was also evaluated. The levels of TNF (both intracellular and extracellular) increased after LPS exposure. Other studies have previously demonstrated that TNF is increased in microglial cells after LPS stimulation and may participate in retinal neovascularization (He et al., 2002; Yoshida et al., 2004). There is evidence indicating that astrocytes and Müller cells can also play an important role in neuroinflammation, releasing TNF in response to bacterial insult (Chung and Benveniste, 1990; Kumar et al., 2013). Therefore, the contribution of these cells to the increased levels of TNF cannot be discarded. Nevertheless, concerning TNF levels, the treatment with sitagliptin, in the absence or the presence of the Y_1 receptor antagonist, did not affect the increase in TNF production triggered by LPS, suggesting that the potential anti-inflammatory effects of sitagliptin appear to be selective, affecting specific molecules such as iNOS.

As for TNF, IL-1β upregulation in the retina and retinal vessels of diabetic rats has previously been described (Liu et al., 2012). In the present study, exposure to LPS increased the production and release of IL-1β in primary retinal neural cell cultures. There was a tendency for an inhibitory effect of sitagliptin, since

the increase in IL-1β extracellular levels, but not intracellular levels, triggered by LPS, was diminished in the presence of sitagliptin, although not statistically significant, further suggesting that sitagliptin does not appear to affect significantly the production of cytokines. Moreover, the presence of the Y_1 receptor antagonist did not affect the production of IL-1β, which is not in agreement with previous findings showing that the activation of Y_1 receptor by NPY inhibits LPS-induced IL-1β release (Ferreira et al., 2010). However, Ferreira and colleagues used a cell line, and we are using mixed primary retinal cell cultures, which are two very distinct biological systems, where the expression of NPY receptors can be substantially different. It must be also emphasized that DPP-IV inhibition does not affect exclusively the NPY system. For instance, inhibiting DPP-IV prolongs the half-life of endogenously released GLP-1 (Ahren and Schmitz, 2004), which has been described to prevent LPS-induced IL-1β production in cultured rat astrocytes (Iwai et al., 2006). Since we did not detect any significant effect of sitagliptin on IL-1β levels, it seems that endogenous GLP-1, as the NPY system, also does not have a particular role in modulating the production of IL-1β in retinal cell cultures exposed to LPS.

As mentioned above, sitagliptin presented a tendency to inhibit the increase of IL-1β levels triggered by LPS, and so we further evaluated a potential effect of sitagliptin on IL-1β production, by analyzing the immunoreactivity of this cytokine. IL-1β-IR was clearly upregulated upon LPS exposure, and sitagliptin seems to attenuate this effect, which is somehow consistent with the inhibitory tendency caused by sitagliptin on IL-1β levels detected by ELISA. Moreover, these immunocytochemistry experiments allowed us concluding that this cytokine is being mainly produced and released by microglial cells. This effect of LPS in microglial cells has previously been described (Kim et al., 2004).

Taken together, our findings indicate that sitagliptin is able to inhibit retinal microglia activation, as evidenced by its capacity to inhibit microglial morphological changes triggered by LPS as well as the upregulation in iNOS-IR in microglial cells and iNOS-derived NO levels, which might explain the protective effects detected previously in the retina of diabetic animal models (Goncalves et al., 2012; Goncalves et al., 2014). However, sitagliptin was unable to significantly inhibit the upregulation of pro-inflammatory cytokines triggered by LPS, despite there was a tendency for the inhibition of IL-1β.

Moreover, the results also indicate that the NPY system, and particularly the Y_1 receptors, do not have a relevant contribution for some of the inhibitory effects of sitagliptin detected. Therefore, despite evidences showing clear anti-inflammatory effects of sitagliptin, directly in retinal microglia, it is likely that sitagliptin can only affect some pro-inflammatory pathways in microglia, and so the exact mechanisms involved in these protective effects against inflammation remain to be clarified.

Chapter 5

Conclusion

5. Conclusion

With the results obtained in the present study, we demonstrated that:

- Sitagliptin prevented the alterations in microglia morphology triggered by LPS. Despite a tendency for a potential involvement of the Y_1 receptor on the effects of sitagliptin, the lack of significance does not allow concluding that the effect of sitagliptin is partially dependent on Y_1 receptor activation;
- Sitagliptin significantly inhibited the LPS-induced increase in iNOS immunoreactvity in both primary retinal neural cell cultures and retinal organotypic cultures. The inhibitory effects of sitagliptin were not significantly altered by the blockade of Y_1 receptor;
- Sitagliptin significantly inhibited the increase in NO production triggered by LPS in primary retinal neural cell cultures, and this inhibitory effect appears to be mediated by Y_1 receptor activation;
- Sitagliptin did not prevent the increase in iNOS mRNA expression triggered by LPS in retinal organotypic cultures. However, pre-treatment with Y_1 receptor antagonist enhanced the effect of LPS, suggesting that basal activation of Y_1 receptor by endogenous NPY might have a role in regulating iNOS expression;
- Neither sitagliptin nor Y_1 receptor antagonist affected the increase in extracellular and intracellular TNF levels triggered by LPS in primary retinal neural cell cultures;
- Neither sitagliptin nor Y_1 receptor antagonist significantly affected the increase in extracellular and intracellular TNF levels triggered by LPS in primary retinal neural cell cultures. However, preliminary results indicate that sitagliptin was able to attenuate the LPS-induced increase of IL-1β immunoreactivity in primary retinal neural cell cultures.

In summary, this study clearly showed that sitagliptin has anti-inflammatory properties, exerting direct inhibitory effects on retinal microglia activation, as demonstrated by the inhibition of a) alterations on microglia morphology, b) increase in iNOS immunoreactivity in retinal microglia and NO production, c) increase in IL-1β immunoreactivity in retinal microglia, triggered by LPS.

Chapter 6

References

6. References

Abid, K., Rochat, B., Lassahn, P.G., Stocklin, R., Michalet, S., Brakch, N., Aubert, J.F., Vatansever, B., Tella, P., De Meester, I., *et al.* (2009). Kinetic study of neuropeptide Y (NPY) proteolysis in blood and identification of NPY3-35: a new peptide generated by plasma kallikrein. The Journal of biological chemistry *284*, 24715-24724.

Abu El-Asrar, A.M. (2013). Evolving strategies in the management of diabetic retinopathy. Middle East African journal of ophthalmology *20*, 273-282.

Adrian, T.E., Bacarese-Hamilton, A.J., Smith, H.A., Chohan, P., Manolas, K.J., and Bloom, S.R. (1987). Distribution and postprandial release of porcine peptide YY. The Journal of endocrinology *113*, 11-14.

Ahren, B., and Schmitz, O. (2004). GLP-1 receptor agonists and DPP-4 inhibitors in the treatment of type 2 diabetes. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme *36*, 867-876.

Alvaro, A.R., Rosmaninho-Salgado, J., Ambrosio, A.F., and Cavadas, C. (2009). Neuropeptide Y inhibits [Ca2+]i changes in rat retinal neurons through NPY Y1, Y4, and Y5 receptors. Journal of neurochemistry *109*, 1508-1515.

Alvaro, A.R., Rosmaninho-Salgado, J., Santiago, A.R., Martins, J., Aveleira, C., Santos, P.F., Pereira, T., Gouveia, D., Carvalho, A.L., Grouzmann, E., *et al.* (2007). NPY in rat retina is present in neurons, in endothelial cells and also in microglial and Muller cells. Neurochemistry international *50*, 757-763.

Amirpour, N., Karamali, F., Razavi, S., Esfandiari, E., and Nasr-Esfahani, M.H. (2014). A proper protocol for isolation of retinal pigment epithelium from rabbit eyes. Advanced biomedical research *3*, 4.

Andersen, C.L., Jensen, J.L., and Orntoft, T.F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer research *64*, 5245-5250.

Antonetti, D.A., Barber, A.J., Bronson, S.K., Freeman, W.M., Gardner, T.W., Jefferson, L.S., Kester, M., Kimball, S.R., Krady, J.K., LaNoue, K.F., *et al.* (2006). Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. Diabetes *55*, 2401-2411.

Barber, A.J. (2003). A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Progress in neuro-psychopharmacology & biological psychiatry *27*, 283-290.

Barber, A.J., Lieth, E., Khin, S.A., Antonetti, D.A., Buchanan, A.G., and Gardner, T.W. (1998). Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. The Journal of clinical investigation *102*, 783-791.

Bedoui, S., von Horsten, S., and Gebhardt, T. (2007). A role for neuropeptide Y (NPY) in phagocytosis: implications for innate and adaptive immunity. Peptides *28*, 373-376.

Berglund, M.M., Hipskind, P.A., and Gehlert, D.R. (2003). Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. Experimental biology and medicine *228*, 217-244.

Bruun, A., and Ehinger, B. (1993). NPY-induced neurotransmitter release from the rabbit and chicken retina. Acta ophthalmologica *71*, 590-596.

Canto Soler, M.V., Gallo, J.E., Dodds, R.A., Hokfelt, T., Villar, M.J., and Suburo, A.M. (2002). Y1 receptor of neuropeptide Y as a glial marker in proliferative vitreoretinopathy and diseased human retina. Glia *39*, 320-324.

Chang, S.Y., Kim, D.B., Ryu, G.R., Ko, S.H., Jeong, I.K., Ahn, Y.B., Jo, Y.H., and Kim, M.J. (2013). Exendin-4 inhibits iNOS expression at the protein level in LPS-stimulated Raw264.7 macrophage by the activation of cAMP/PKA pathway. Journal of cellular biochemistry *114*, 844-853.

Chung, I.Y., and Benveniste, E.N. (1990). Tumor necrosis factor-alpha production by astrocytes. Induction by lipopolysaccharide, IFN-gamma, and IL-1 beta. Journal of immunology *144*, 2999-3007.

D'Angelo, I., Oh, S.J., Chun, M.H., and Brecha, N.C. (2002). Localization of neuropeptide Y1 receptor immunoreactivity in the rat retina and the synaptic connectivity of Y1 immunoreactive cells. The Journal of comparative neurology *454*, 373-382.

Dimayuga, F.O., Wang, C., Clark, J.M., Dimayuga, E.R., Dimayuga, V.M., and Bruce-Keller, A.J. (2007). SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. Journal of neuroimmunology *182*, 89-99.

Dimitrijevic, M., and Stanojevic, S. (2013). The intriguing mission of neuropeptide Y in the immune system. Amino acids *45*, 41-53.

Dimitrijevic, M., Stanojevic, S., Mitic, K., Kustrimovic, N., Vujic, V., Miletic, T., and Kovacevic-Jovanovic, V. (2008). The anti-inflammatory effect of neuropeptide Y (NPY) in rats is dependent on dipeptidyl peptidase 4 (DP4) activity and age. Peptides *29*, 2179-2187.

Dowler, J.G. (2003). Laser management of diabetic retinopathy. Journal of the Royal Society of Medicine *96*, 277-279.

Drucker, D.J., and Nauck, M.A. (2006). The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet *368*, 1696-1705.

Dumont, Y., Fournier, A., and Quirion, R. (1998). Expression and characterization of the neuropeptide Y Y5 receptor subtype in the rat brain. The Journal of neuroscience : the official journal of the Society for Neuroscience *18*, 5565-5574.

Ekblad, E., Edvinsson, L., Wahlestedt, C., Uddman, R., Hakanson, R., and Sundler, F. (1984). Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibers. Regulatory peptides *8*, 225-235.

Ferreira, R., Santos, T., Cortes, L., Cochaud, S., Agasse, F., Silva, A.P., Xapelli, S., and Malva, J.O. (2012). Neuropeptide Y inhibits interleukin-1 beta-induced microglia motility. Journal of neurochemistry *120*, 93-105.

Ferreira, R., Santos, T., Viegas, M., Cortes, L., Bernardino, L., Vieira, O.V., and Malva, J.O. (2011). Neuropeptide Y inhibits interleukin-1beta-induced phagocytosis by microglial cells. Journal of neuroinflammation *8*, 169.

Ferreira, R., Xapelli, S., Santos, T., Silva, A.P., Cristovao, A., Cortes, L., and Malva, J.O. (2010). Neuropeptide Y modulation of interleukin-1{beta} (IL-1{beta})-induced nitric oxide production in microglia. The Journal of biological chemistry *285*, 41921-41934.

Fiebich, B.L., Butcher, R.D., and Gebicke-Haerter, P.J. (1998). Protein kinase C-mediated regulation of inducible nitric oxide synthase expression in cultured microglial cells. Journal of neuroimmunology *92*, 170-178.

Fischbarg, J. (2006). Advances in Organ Biology - The Biology of the Eye Vol 10 (USA: Elsevier).

Fong, D.S., Aiello, L., Gardner, T.W., King, G.L., Blankenship, G., Cavallerano, J.D., Ferris, F.L., 3rd, Klein, R., and American Diabetes, A. (2004). Retinopathy in diabetes. Diabetes care *27 Suppl 1*, S84-87.

Frost-Larsen, K., Larsen, H.W., and Simonsen, S.E. (1980). Oscillatory potential and nyctometry in insulin-dependent diabetics. Acta ophthalmologica *58*, 879-888.

Funkelstein, L., Lu, W.D., Koch, B., Mosier, C., Toneff, T., Taupenot, L., O'Connor, D.T., Reinheckel, T., Peters, C., and Hook, V. (2012). Human cathepsin V protease participates in production of enkephalin and NPY neuropeptide neurotransmitters. The Journal of biological chemistry *287*, 15232-15241.

Funkelstein, L., Toneff, T., Hwang, S.R., Reinheckel, T., Peters, C., and Hook, V. (2008). Cathepsin L participates in the production of neuropeptide Y in secretory vesicles, demonstrated by protease gene knockout and expression. Journal of neurochemistry *106*, 384-391.

Garden, G.A., and Moller, T. (2006). Microglia biology in health and disease. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *1*, 127-137.

Garg, K., Tripathi, C.D., and Kumar, S. (2013). Clinical review of sitagliptin: a DPP-4 inhibitor. The Journal of the Association of Physicians of India *61*, 645-649.

Gavet, O., and Pines, J. (2010). Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. Developmental cell *18*, 533-543.

Goncalves, A., Leal, E., Paiva, A., Teixeira Lemos, E., Teixeira, F., Ribeiro, C.F., Reis, F., Ambrosio, A.F., and Fernandes, R. (2012). Protective effects of the dipeptidyl peptidase IV inhibitor sitagliptin in the blood-retinal barrier in a type 2 diabetes animal model. Diabetes, obesity & metabolism *14*, 454-463.

Goncalves, A., Marques, C., Leal, E., Ribeiro, C.F., Reis, F., Ambrosio, A.F., and Fernandes, R. (2014). Dipeptidyl peptidase-IV inhibition prevents blood-retinal barrier breakdown, inflammation and neuronal cell death in the retina of type 1 diabetic rats. Biochimica et biophysica acta *1842*, 1454-1463.

Grandt, D., Schimiczek, M., Rascher, W., Feth, F., Shively, J., Lee, T.D., Davis, M.T., Reeve, J.R., Jr., and Michel, M.C. (1996). Neuropeptide Y 3-36 is an endogenous ligand selective for Y2 receptors. Regulatory peptides *67*, 33-37.

Gray, T.S., and Morley, J.E. (1986). Neuropeptide Y: anatomical distribution and possible function in mammalian nervous system. Life sciences *38*, 389-401.

Gregor, P., Feng, Y., DeCarr, L.B., Cornfield, L.J., and McCaleb, M.L. (1996). Molecular characterization of a second mouse pancreatic polypeptide receptor and its inactivated human homologue. The Journal of biological chemistry *271*, 27776-27781.

He, B.P., Wen, W., and Strong, M.J. (2002). Activated microglia (BV-2) facilitation of TNF-alpha-mediated motor neuron death in vitro. Journal of neuroimmunology *128*, 31-38.

Hosoya, K., and Tomi, M. (2008). Inner Blood—Retinal Barrier: Transport Biology and Methodology. (Springer US).

Iwai, T., Ito, S., Tanimitsu, K., Udagawa, S., and Oka, J. (2006). Glucagon-like peptide-1 inhibits LPS-induced IL-1beta production in cultured rat astrocytes. Neuroscience research *55*, 352-360.

Jen, P.Y., Li, W.W., and Yew, D.T. (1994). Immunohistochemical localization of neuropeptide Y and somatostatin in human fetal retina. Neuroscience *60*, 727-735.

Jotwani, G., Itoh, K., and Wadhwa, S. (1994). Immunohistochemical localization of tyrosine hydroxylase, substance P, neuropeptide-Y and leucine-enkephalin in developing human retinal amacrine cells. Brain research Developmental brain research *77*, 285-289.

Kaneda, M. (2013). Signal processing in the mammalian retina. Journal of Nippon Medical School = Nippon Ika Daigaku zasshi *80*, 16-24.

Karlstetter, M., Ebert, S., and Langmann, T. (2010). Microglia in the healthy and degenerating retina: insights from novel mouse models. Immunobiology *215*, 685-691.

Kaske, M. (2012). In Search for Potent and Selective NPY Y4 Receptor Ligands: Acylguanidines, Argininamides and Peptide Analogs In Chemistry and Pharmacy (University of Regensburg).

Kern, T.S. (2007). Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Experimental diabetes research *2007*, 95103.

Kettenmann, H., Hanisch, U.K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. Physiological reviews *91*, 461-553.

Kierdorf, K., and Prinz, M. (2013). Factors regulating microglia activation. Frontiers in cellular neuroscience *7*, 44.

Kim, S.H., Smith, C.J., and Van Eldik, L.J. (2004). Importance of MAPK pathways for microglial pro-inflammatory cytokine IL-1 beta production. Neurobiology of aging *25*, 431-439.

Kolb, H. (1995). Gross Anatomy of the Eye. In Webvision: The Organization of the Retina and Visual System, H. Kolb, E. Fernandez, and R. Nelson, eds. (Salt Lake City (UT)).

Kumar, A., Pandey, R.K., Miller, L.J., Singh, P.K., and Kanwar, M. (2013). Muller glia in retinal innate immunity: a perspective on their roles in endophthalmitis. Critical reviews in immunology *33*, 119-135.

Kurpius, D., Wilson, N., Fuller, L., Hoffman, A., and Dailey, M.E. (2006). Early activation, motility, and homing of neonatal microglia to injured neurons does not require protein synthesis. Glia *54*, 58-70.

Lang, G.K. (2000). Ophthalmology - A Short Text Book (New York: Thiemes Stuttgart).

Langmann, T. (2007). Microglia activation in retinal degeneration. Journal of leukocyte biology *81*, 1345-1351.

Larhammar, D. (1996). Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide. Regulatory peptides *62*, 1-11.

Leal, E.C., Manivannan, A., Hosoya, K., Terasaki, T., Cunha-Vaz, J., Ambrosio, A.F., and Forrester, J.V. (2007). Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. Investigative ophthalmology & visual science *48*, 5257-5265.

Lee, J.E., Liang, K.J., Fariss, R.N., and Wong, W.T. (2008). Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. Investigative ophthalmology & visual science *49*, 4169-4176.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J., and Vartanian, T. (2003). Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proceedings of the National Academy of Sciences of the United States of America *100*, 8514-8519.

Lieb, K., Engels, S., and Fiebich, B.L. (2003). Inhibition of LPS-induced iNOS and NO synthesis in primary rat microglial cells. Neurochemistry international *42*, 131-137.

Liu, Y., Biarnes Costa, M., and Gerhardinger, C. (2012). IL-1beta is upregulated in the diabetic retina and retinal vessels: cell-specific effect of high glucose and IL-1beta autostimulation. PloS one *7*, e36949.

Lundell, I., Blomqvist, A.G., Berglund, M.M., Schober, D.A., Johnson, D., Statnick, M.A., Gadski, R.A., Gehlert, D.R., and Larhammar, D. (1995). Cloning of a human receptor of the NPY receptor family with high affinity for pancreatic polypeptide and peptide YY. The Journal of biological chemistry *270*, 29123-29128.

Matsubara, H., Kuze, M., Sasoh, M., Ma, N., Furuta, M., and Uji, Y. (2006). Time-dependent course of electroretinograms in the spontaneous diabetic Goto-Kakizaki rat. Japanese journal of ophthalmology *50*, 211-216.

Mentlein, R. (1999). Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regulatory peptides *85*, 9-24.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998). XVI. International Union of Pharmacology

recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacological reviews *50*, 143-150.

Minth, C.D., Bloom, S.R., Polak, J.M., and Dixon, J.E. (1984). Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. Proceedings of the National Academy of Sciences of the United States of America *81*, 4577-4581.

Morris, S.M., Jr., and Billiar, T.R. (1994). New insights into the regulation of inducible nitric oxide synthesis. The American journal of physiology *266*, E829-839.

Nam Han Cho, D.W., Leonor Guariguata, P.A.M., Nita, Forouhi, I.H., Rui Li, Azeem Majeed,, Jean Claude Mbanya, A.M., K.M. Venkat, Narayan, A.R., Wolfgang, Rathmann, G.R., Jonathan Shaw, Martin, and Silink, D.R.R.W., Ping Zhang (2013). IDF Diabetes Atlas, 6th edition edn.

Nave, H., Bedoui, S., Moenter, F., Steffens, J., Felies, M., Gebhardt, T., Straub, R.H., Pabst, R., Dimitrijevic, M., Stanojevic, S., *et al.* (2004). Reduced tissue immigration of monocytes by neuropeptide Y during endotoxemia is associated with Y2 receptor activation. Journal of neuroimmunology *155*, 1-12.

Newman, E., and Reichenbach, A. (1996). The Muller cell: a functional element of the retina. Trends in neurosciences *19*, 307-312.

Oh, S.J., D'Angelo, I., Lee, E.J., Chun, M.H., and Brecha, N.C. (2002). Distribution and synaptic connectivity of neuropeptide Y-immunoreactive amacrine cells in the rat retina. The Journal of comparative neurology *446*, 219-234.

Parker, R.M., and Herzog, H. (1999). Regional distribution of Y-receptor subtype mRNAs in rat brain. The European journal of neuroscience *11*, 1431-1448.

Pedrazzini, T., Pralong, F., and Grouzmann, E. (2003). Neuropeptide Y: the universal soldier. Cellular and molecular life sciences : CMLS *60*, 350-377.

Purves, D. (2004). Neuroscience, Third edition edn (Sunderland, Massachusetts U.S.A.: Sinauer Associates, Inc.).

Saha, R.N., and Pahan, K. (2006). Signals for the induction of nitric oxide synthase in astrocytes. Neurochemistry international *49*, 154-163.

Santiago, A.R., Baptista, F.I., Santos, P.F., Cristóvão, G., Ambrosio, A.F., Cunha, R.A., and Gomes, C.A. (2014). Role of Microglia Adenosine A_{2A} Receptors in Retinal and Brain Neurodegenerative Diseases. Mediators of Inflammation *2014*, 13.

Santiago, A.R., Pereira, T.S., Garrido, M.J., Cristovao, A.J., Santos, P.F., and Ambrosio, A.F. (2006). High glucose and diabetes increase the release of [3H]-D-aspartate in retinal cell cultures and in rat retinas. Neurochemistry international *48*, 453-458.

Santos-Carvalho, A., Alvaro, A.R., Martins, J., Ambrosio, A.F., and Cavadas, C. (2014). Emerging novel roles of neuropeptide Y in the retina: from neuromodulation to neuroprotection. Progress in neurobiology *112*, 70-79.

Santos-Carvalho, A., Aveleira, C.A., Elvas, F., Ambrosio, A.F., and Cavadas, C. (2013). Neuropeptide Y receptors Y1 and Y2 are present in neurons and glial cells in rat retinal cells in culture. Investigative ophthalmology & visual science *54*, 429-443.

Schuetz, E., and Thanos, S. (2004). Microglia-targeted pharmacotherapy in retinal neurodegenerative diseases. Current drug targets *5*, 619-627.

Seki, M., Tanaka, T., Nawa, H., Usui, T., Fukuchi, T., Ikeda, K., Abe, H., and Takei, N. (2004). Involvement of brain-derived neurotrophic factor in early retinal neuropathy of streptozotocin-induced diabetes in rats: therapeutic potential of brain-derived neurotrophic factor for dopaminergic amacrine cells. Diabetes *53*, 2412-2419.

Shaw, J.L., Gackenheimer, S.L., and Gehlert, D.R. (2003). Functional autoradiography of neuropeptide Y Y1 and Y2 receptor subtypes in rat brain using agonist stimulated [35S]GTPgammaS binding. Journal of chemical neuroanatomy *26*, 179-193.

Sheetz, M.J., and King, G.L. (2002). Molecular understanding of hyperglycemia's adverse effects for diabetic complications. JAMA : the journal of the American Medical Association *288*, 2579-2588.

Simpson, K., Parker, J., Plumer, J., and Bloom, S. (2012). CCK, PYY and PP: the control of energy balance. Handbook of experimental pharmacology, 209-230.

Sinclair, J.R., Jacobs, A.L., and Nirenberg, S. (2004). Selective ablation of a class of amacrine cells alters spatial processing in the retina. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 1459-1467.

Sinclair, J.R., and Nirenberg, S. (2001). Characterization of neuropeptide Y-expressing cells in the mouse retina using immunohistochemical and transgenic techniques. The Journal of comparative neurology *432*, 296-306.

Siu, T.L., Morley, J.W., and Coroneo, M.T. (2008). Toxicology of the retina: advances in understanding the defence mechanisms and pathogenesis of drug- and light-induced retinopathy. Clinical & experimental ophthalmology *36*, 176-185.

Tarr, J.M., Kaul, K., Chopra, M., Kohner, E.M., and Chibber, R. (2013). Pathophysiology of diabetic retinopathy. ISRN ophthalmology *2013*, 343560.

Tatemoto, K. (1982). Neuropeptide Y: complete amino acid sequence of the brain peptide. Proceedings of the National Academy of Sciences of the United States of America *79*, 5485-5489.

Uckermann, O., Wolf, A., Kutzera, F., Kalisch, F., Beck-Sickinger, A.G., Wiedemann, P., Reichenbach, A., and Bringmann, A. (2006). Glutamate release by neurons evokes a purinergic inhibitory mechanism of osmotic glial cell swelling in the rat retina: activation by neuropeptide Y. Journal of neuroscience research *83*, 538-550.

Wang, M., Ma, W., Zhao, L., Fariss, R.N., and Wong, W.T. (2011). Adaptive Muller cell responses to microglial activation mediate neuroprotection and coordinate inflammation in the retina. Journal of neuroinflammation *8*, 173.

Weinberg, D.H., Sirinathsinghji, D.J., Tan, C.P., Shiao, L.L., Morin, N., Rigby, M.R., Heavens, R.H., Rapoport, D.R., Bayne, M.L., Cascieri, M.A*., et al.* (1996). Cloning and expression of a novel neuropeptide Y receptor. The Journal of biological chemistry *271*, 16435-16438.

Wettstein, J.G., Earley, B., and Junien, J.L. (1995). Central nervous system pharmacology of neuropeptide Y. Pharmacology & therapeutics *65*, 397-414.

Wolak, M.L., DeJoseph, M.R., Cator, A.D., Mokashi, A.S., Brownfield, M.S., and Urban, J.H. (2003). Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry. The Journal of comparative neurology *464*, 285-311.

Yau, J.W., Rogers, S.L., Kawasaki, R., Lamoureux, E.L., Kowalski, J.W., Bek, T., Chen, S.J., Dekker, J.M., Fletcher, A., Grauslund, J*., et al.* (2012). Global prevalence and major risk factors of diabetic retinopathy. Diabetes care *35*, 556-564.

Yoshida, S., Yoshida, A., and Ishibashi, T. (2004). Induction of IL-8, MCP-1, and bFGF by TNF-alpha in retinal glial cells: implications for retinal neovascularization during post-ischemic inflammation. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie *242*, 409-413.

Zeng, H.Y., Green, W.R., and Tso, M.O. (2008). Microglial activation in human diabetic retinopathy. Archives of ophthalmology *126*, 227-232.

Zhang, G., He, J.L., Xie, X.Y., and Yu, C. (2012). LPS-induced iNOS expression in N9 microglial cells is suppressed by geniposide via ERK, p38 and nuclear factor-kappaB signaling pathways. International journal of molecular medicine *30*, 561-568.

Zhang, W., Liu, H., Rojas, M., Caldwell, R.W., and Caldwell, R.B. (2011). Anti-inflammatory therapy for diabetic retinopathy. Immunotherapy *3*, 609-628.