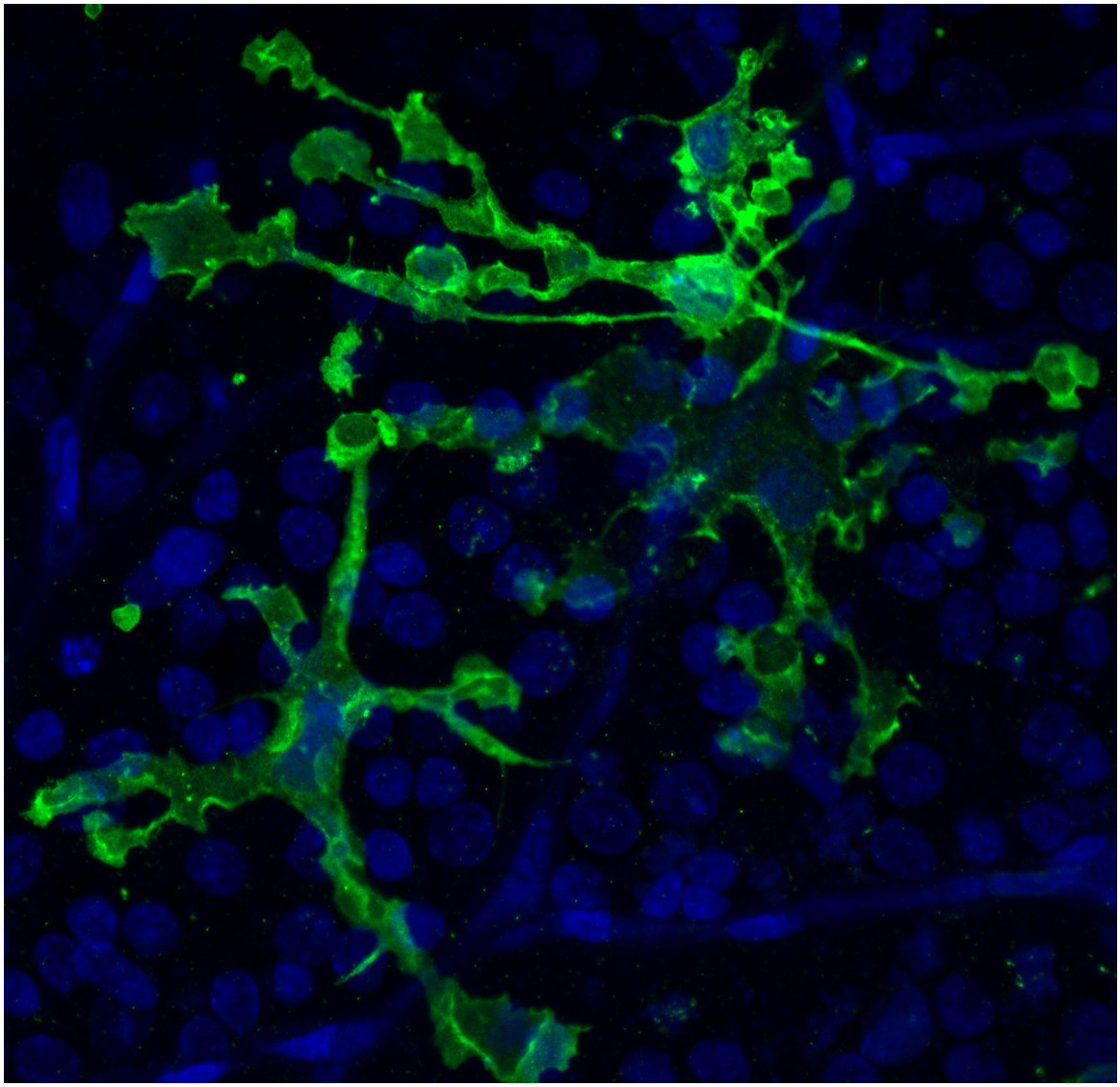

Filipe Manuel Rijo Mendes Martins Elvas

Modulation of pro-inflammatory responses in the retina by Neuropeptide Y – the role of NPY Y_1 receptor

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Farmacologia Aplicada. O trabalho foi realizado sob a orientação científica do Investigador Doutor António Francisco Rosa Gomes Ambrósio (Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina da Universidade de Coimbra) e supervisão da Professora Doutora Cláudia Margarida Gonçalves Cavadas (Faculdade de Farmácia da Universidade de Coimbra).

Setembro de 2013

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UNIVERSIDADE DE COIMBRA

On the front page:

Microglial cells in cultured retinal explants, expressing CD11b (in green). Nuclear staining in blue (DAPI staining).

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Universidade de Coimbra

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Objectives

Retinal degenerative diseases, such as glaucoma and diabetic retinopathy, are the main cause of irreversible vision loss worldwide. In the retina, microglia activation has been shown to be associated with glaucoma and diabetic retinopathy (Yuan and Neufeld, 2001, Zeng et al., 2008, Bosco et al., 2011), potentially contributing for the progression of retinal degenerative diseases.

Neuropeptide Y is a neurotransmitter and neuromodulator that exerts its effects via four G protein-coupled receptors (Y_1R, Y_2R, Y_4R and Y_5R). Several evidences suggest that NPY has a neuroprotective role in models of CNS injury, including the retina. Moreover, it was also demonstrated an important role of NPY in the modulation of the functions of immune cells. However, the mechanisms by which NPY can be neuroprotective in retinal degenerative diseases in not completely elucidated. It has been postulated that these mechanisms can involve the triggering of antiapoptotic pathways, inhibition of excitotoxic cell death or modulation of microglia pro-inflammatory responses.

In this project, we aimed at evaluating whether the manipulation of the NPY system could inhibit pro-inflammatory processes in the retina, especially controlling retinal microglia reactivity, giving a particular attention to the Y_1R. In order to achieve this goal, first we used *in vitro* models, exposing retinal microglial cells to a pro-inflammatory stimulus (LPS) in three different culture preparations: cultured retinal explants, primary retinal mixed cultures and purified cultures of retinal microglial cells. Second, we used an animal model of retinal degeneration, retinal ischemia-reperfusion injury model. Several markers of the retinal inflammatory status were evaluated in the absence or presence of NPY or an Y_1 receptor agonist and/or antagonist, such as retinal microglia activation in terms of morphology, inducible protein expression and reactive oxygen species production, and pro-inflammatory cytokine expression and production in the retina.

Abstract

Neuropeptide Y (NPY) is a 36 amino acid peptide that is abundantly distributed in the central nervous system (CNS), including the retina. NPY acts through the activation of several G protein-coupled receptors: Y_1R, Y_2R, Y_4R and Y_5R. It has been shown that this peptide has neuromodulatory and neuroprotective roles in the retina, and it has been associated with physiological and pathological conditions. Increasing evidence has shown that NPY is a regulator of inflammatory processes, but its effects depend on cell types and tissues, the type of NPY receptors involved and on factors present in the cellular milieu. However, little is known about its potential inhibitory effects on pro-inflammatory processes in the retina, especially controlling retinal microglia reactivity.

Microglia are the innate immune cells of the CNS and are involved in the maintenance of retinal homeostasis. However, in response to retinal injury, activated microglia adopt ameboid morphology, express inducible nitric oxide synthase (iNOS) and release neurotoxic factors such as the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and reactive oxygen species (ROS), which can lead to neuronal degeneration. The detrimental effects of overactivated microglia are thought to contribute to the pathogenesis retinal degenerative diseases, such as diabetic retinopathy and glaucoma.

Since neuroinflammation is described to be involved in the pathogenesis of several retinal degenerative diseases, we investigated the NPY effects, particularly via the Y_1R activation, on the modulation of microglia activation and inhibition of pro-inflammatory processes in the retina.

To induce an inflammatory response, cultured retinal explants, primary retinal neural mixed cultures and purified cultures of retinal microglial cells were exposed to lipopolysaccharide (LPS), in the absence or presence of NPY or a Y_1 receptor agonist ([Leu^{31}, Pro^{34}]-NPY) and/or antagonist (BIBP3226). Additionally, an animal model of retinal degeneration, a retinal ischemia-reperfusion (I/R) injury model, was used. In this case, NPY or [Leu^{31}, Pro^{34}]-NPY (LP-NPY) were injected intravitreally 1 h before ischemia and retinal blood flow was restored for 8 h or 24 h. Several markers and parameters of the retinal inflammatory status were evaluated, including the activation of retinal microglial cells, in terms of changes in morphology and inducible protein expression, as well as the expression and production of pro-inflammatory cytokines in the retina.

In cultured retinal explants, NPY was able to inhibit the alterations in retinal microglia morphology, as well as the increase in iNOS expression and ROS production in retinal microglia, triggered by LPS. Moreover, NPY inhibited IL-1β and IL-6 expression and

production. Y_1R activation mimicked the inhibitory effects of NPY on the LPS-induced alterations in retinal microglia morphology and iNOS expression. In addition, activation of Y_1R inhibited the expression and production of all pro-inflammatory cytokines studied (TNF-α, IL-1β and IL-6), indicating that Y_1R appears to have a predominant role on the effects mediated by NPY.

In the I/R injury model, intravitreal injection of NPY or LP-NPY before ischemia inhibited morphological changes in retinal microglia induced by I/R 24 h after reperfusion. Furthermore, 8 h after reperfusion the upregulation of TNF-α, IL-1β and IL-6, and the production of TNF-α and IL-6, in ischemic retinas, was inhibited by NPY.

Altogether, these data provide evidence that NPY and Y_1R activation are able to regulate retinal microglia activation and inhibit the expression and production of neurotoxic factors in the retina. Immunohistochemistry data and in vitro studies indicate that retinal microglial cells primarily express iNOS and are the primary sources of ROS production under pro-inflammatory conditions in the retina. These findings could point novel physiological and therapeutic roles of NPY system in neuroinflammation, not only in the retina, but also in the nervous system.

Resumo

O neuropeptídeo Y (NPY) é um peptídeo com 36 aminoácidos que se encontra amplamente distribuído no sistema nervoso central (SNC), incluindo a retina. Os seus efeitos são mediados através da ativação de vários recetores acoplados a proteínas G: Y_1R, Y_2R, Y_4R e Y_5R e y_6R. Este peptídeo pode atuar como neuromodulador e neuroprotetor na retina, e tem sido associado a diversas doenças e processos fisiológicos. Evidências crescentes têm demonstrado que o NPY é um regulador de processos inflamatórios, dependendo os seus efeitos do tipo de tecidos e células em que atua, do tipo de recetores envolvidos e dos fatores presentes no meio. No entanto, o seu potencial papel anti-inflamatório na retina, em particular no controlo da reatividade da microglia, é praticamente desconhecido.

As células da microglia são células do sistema imunitário do SNC, e têm um papel na homeostase da retina. No entanto, em resposta a lesões na retina, as células da microglia ficam ativadas, adotando uma morfologia ameboide, expressam a isoforma indutível da sintase do monóxido de azoto (iNOS), libertam substâncias neurotóxicas, como por exemplo as citocinas pró-inflamatórias TNF-α, IL-1β e IL-6, e espécies reativas de oxigénio (ROS), o que pode contribuir para a morte neuronal. Pensa-se que os efeitos nocivos da microglia ativada podem contribuir para a patogénese de doenças degenerativas da retina, tais como a retinopatia diabética e o glaucoma.

Uma vez que se considera que a neuroinflamação está envolvida na patogénese de várias doenças da retina, neste trabalho investigou-se os efeitos do NPY, e em particular da ativação do recetor Y_1 (Y_1R), na modulação da ativação da microglia e na inibição da resposta pro-inflamatória na retina.

Para induzir uma resposta inflamatória, expuseram-se culturas de explantes de retina, culturas mistas de retina e culturas purificadas de microglia de retina a lipopolissacarídeo (LPS), na ausência ou presença de NPY ou de um agonista ([Leu^{31}, Pro^{34}]-NPY) e/ou antagonista (BIBP3226) do Y_1R. Adicionalmente, foi utilizado um modelo animal de isquémia-reperfusão (I/R) da retina. Neste caso, o NPY ou o [Leu^{31}, Pro^{34}]-NPY (LP-NPY) foram injetados no vítreo 1 h antes da isquémia, tendo sido utilizados dois tempos de reperfusão, 8 e 24 h. Foram avaliados diversos marcadores e parâmetros indicadores de inflamação na retina, incluindo o estado de ativação das células da microglia, em termos de alterações morfológicas, assim como a expressão e produção de citocinas pro-inflamatórias.

Em culturas de explantes de retina, o NPY inibiu as alterações na morfologia das células da microglia, bem como o aumento da expressão de iNOS e a produção de ROS na

microglia de retina, desencadeados pela exposição a LPS. Por outro lado, o NPY inibiu a expressão e produção de IL-1β e IL-6. A ativação do Y_1R mimetizou os efeitos do NPY na inibição das alterações morfológicas e expressão de iNOS na microglia de retina induzidas pelo LPS. Além disso, a ativação do Y_1R inibiu a expressão e produção de todas as citocinas pró-inflamatórias estudadas (TNF-α, IL-1β e IL-6), sugerindo um papel importante para o Y_1R nos efeitos mediados pelo NPY.

No modelo de I/R, a injeção intravítrea de NPY ou de LP-NPY antes da isquémia inibiu as alterações morfológicas nas células da microglia de retina induzidas pela I/R após 24 h de reperfusão. Além disso, após 8 h de reperfusão, o aumento na expressão de TNF-α, IL-1β e IL-6 e na produção de TNF-α e IL-6 em retinas sujeitas a isquémia foi inibido pelo NPY.

No seu conjunto, os dados obtidos revelam que o NPY e a ativação do Y1R são capazes de regular a ativação da microglia de retina e inibir a expressão e produção de fatores neurotóxicos na retina. Estes resultados poderão contribuir para elucidar potenciais efeitos fisiológicos e terapêuticos do NPY em processos neuroinflamatórios não somente na retina, mas também no sistema nervoso.

Contents

Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
ANOVA	Analysis of variance
AU	Arbitrary units
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BRB	Blood retinal barrier
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Ct	Threshold cycle
CX3CL1	Fractalkine
DAPI	4',6' diamino-2-phenylindole
DHE	Dihydroethidium
DIV	Days in vitro
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
RGCL	Retinal ganglion cell layer
GFAP	Glial fibrillary acidic protein
GPF	Green fluorescent protein
HBSS	Hank's Balanced Salt Solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
I/R	Ischemia reperfusion
IL	Interleukin
INL	Inner nuclear layer
iNOS	Inducible nitric-oxide synthase
IOP	Intraocular pressure
IPL	Inner plexiform layer

IR	Immunoreactivity
LP-NPY	[Leu^{31}, Pro^{34}]-NPY
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MDMA	3,4-methylenedioxy-N-methylamphetamine
MEM	Eagle's minimum essential medium
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFL	Nerve fiber layer
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NPY	Neuropeptide Y
NPYRs	NPY receptors
OCT	Optimal cutting temperature compound
ON	Optic nerve
ONH	Optic nerve head
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase C
PP	Pancreatic polypeptide
PRRs	Pattern recognition receptors
PYY	Peptide YY
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RGCs	Retinal ganglion cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Room temperature
TAE	Tris-Acetate-EDTA buffer
TGF-β	Transforming growth factor beta

TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
Y_1R	Neuropeptide Y Y_1 receptor
Y_2R	Neuropeptide Y Y_2 receptor
Y_4R	Neuropeptide Y Y_4 receptor
Y_5R	Neuropeptide Y Y_5 receptor
y_6R	Neuropeptide Y y_6 receptor

INTRODUCTION

1. Introduction

1.1. The Eye

The eye is a complex anatomical structure and the primary organ of vision. It is able to record visual images on the photoreceptors, transform those images in the retina and convey that information to the brain for interpretation and reaction (Blake and Sekuler, 2006, Rogers, 2011).

The human eye is nearly spherical and three concentric layers can be distinguished. The outer region, also called the fibrous tunic, protects the eyeball and consists of the sclera and the cornea. The sclera is a connective tissue coat, which maintains the shape of the eye and protects it against mechanical forces (Galloway et al., 2006, Rogers, 2011). The sclera is covered by a mucous membrane, the conjunctiva. The cornea is an avascular and transparent structure that allows light to enter the eye. It refracts and transmits the light to the lens and retina (Rogers, 2011). These two structures are connected at the limbus. The middle layer of the eye, the vascular tunic, is composed of the choroid, the ciliary body and the iris – the uvea. The choroid is a vascular layer that nourishes the outer retinal layers (Rogers, 2011).

Toward the anterior portion of the eye, the middle layer forms a delicate structure called the ciliary body. This structure is the site of aqueous humor production (Goel et al., 2010), a watery fluid that fills the anterior chamber of the eye located in front of the lens and behind the cornea. It also controls the shape and the optical power of the lens, in a process called accommodation (Blake and Sekuler, 2006). The aqueous humor transports oxygen and nutrients to the cornea and lens, and removes their waste products (Goel et al., 2010). It also helps in the maintenance of a constant pressure inside the eye. However, the ciliary body is constantly producing aqueous humor to prevent the accumulation of waste products and to keep a fresh supply of nourishment (Rogers, 2011). For the pressure to remain constant, a balance between the production and elimination of aqueous fluid must be achieved. Under pathological conditions this equilibrium may be lost and the pressure builds up within the eye (Galloway et al., 2006). Elevated intraocular pressure (IOP) is the major risk factor of glaucoma, the second leading cause of blindness worldwide (Quigley and Broman, 2006).

Away from the wall of the eye, the ciliary body gives rise to the iris. The iris is a pigmented circular structure that controlling the size of the pupil regulates the amount of light reaching the retina (Rogers, 2011). The lens is a transparent structure that lies behind the iris. The elastic capsule over the lens controls the flow of aqueous humor into the lens

(Goel et al., 2010). Along with the cornea it refracts light waves to be focused on the retina (Blake and Sekuler, 2006). The posterior chamber is a triangular space between the back of iris, the lens and the ciliary body, and it is also filled with aqueous humor.

The third eye's chamber, the vitreous chamber, is located behind the lens and in front of the retina. It accounts for about two thirds of the total volume of the eye (Marieb, 2005). This chamber is filled with the vitreous humor, a transparent fluid where debris can accumulate within (Rogers, 2011). The innermost layer of the eye is the retina, consisting of the pigment epithelium, photoreceptors, retinal neurons, glia and blood vessels. The retina is the part of the central nervous system (CNS) that detects light waves and transforms them into messages bound for the brain (Marieb, 2005). The three-layered arrangement of the eye is depicted in Figure 1.

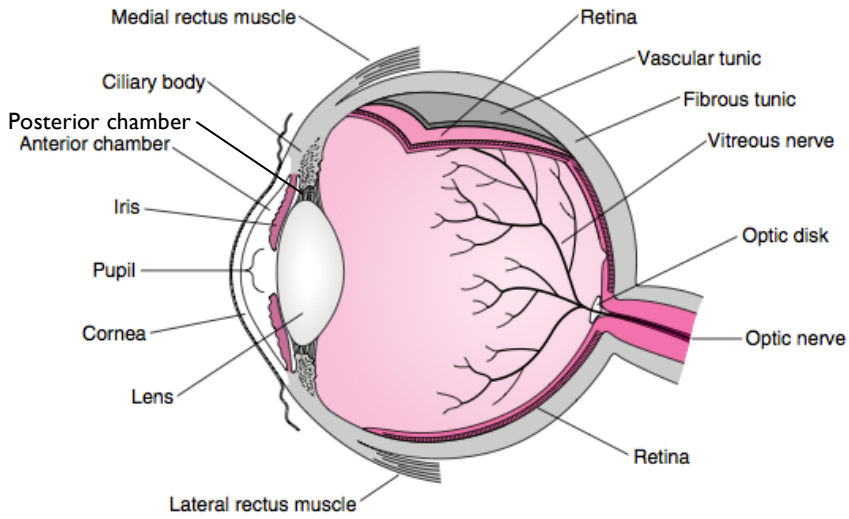

Figure 1. **Cross section of the human eye**, illustrating a three-layered arrangement of the eye, showing the fibrous tunic, vascular tunic and the retina. It also shows the anterior and the posterior chamber, and the main structures of the eye. Adapted from Blake and Sekuler, 2006.

Clinically, the eye can be divided within two segments: anterior segment, encompassing all the structures from the lens forward; and the posterior segment, containing all the structures posterior to the lens (Galloway et al., 2006).

1.1.1. Retina

The retina is the light-sensitive tissue that is located in the back of the eye. It is separated from the choroid by a layer of pigmented cuboidal pigmented cells (Rogers, 2011), known as the retinal pigment epithelium (RPE) (Fig. 2).

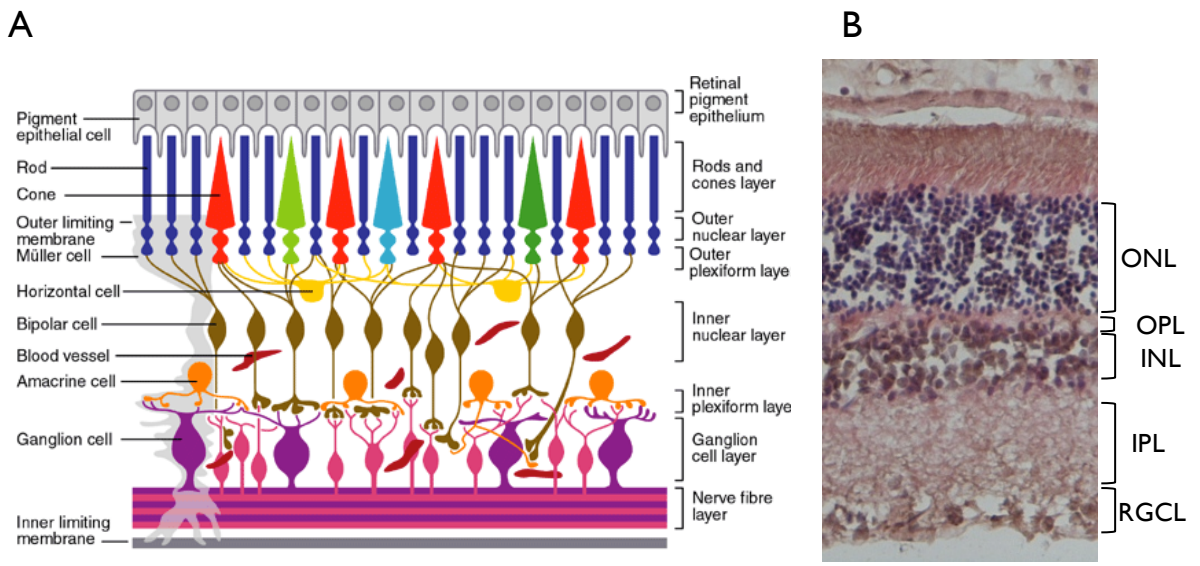

Figure 2. Schematic illustration of the structure of the retina. (A) Cellular organization of the retina. Adapted from Wilkinson-Berka, 2004 (B) Retinal sagittal section of the rat retina stained using hematoxylin and eosin, illustrating the retinal layers. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer.

The RPE is tightly attached to the Bruch's membrane on the choroidal side. Among other functions, the RPE controls the diffusion of particles from the choroid vessels to the retina (la Cour and Ehinger, 2006) and contributes for the regulation of the normal retinal immune status by secreting cytokines, such as transforming growth factor beta (TGF-β) (Langmann, 2007).

Seven layers can be distinguished in the so-called neurosensory retina (Fig. 2). The outermost layer, anterior to the RPE, consists of inner and outer segments of the photoreceptors (rods and cones), the light sensitive cells. The cell bodies of rods and cones form the outer nuclear layer (ONL). The ONL is separated from the photoreceptor segments by the outer limiting membrane (Omri et al., 2010). The outer plexiform layer (OPL) is composed of the synapses between photoreceptors and horizontal and bipolar cells. Microglial cells also populate the OPL (Santos et al., 2008). Next, is located the inner nuclear layer (INL) encompassing the cell bodies of horizontal, bipolar, amacrine and Müller cells. The inner plexiform layer (IPL) consists of the synapses between bipolar, amacrine and retinal ganglion cells (RGCs). IPL also contains microglial cells (Santos et al., 2008). The cell bodies of the RGCs, displaced amacrine cells and astrocytes (Ramirez et al., 1996, Mack et al., 2004) are located in the retinal ganglion cell layer (RGCL). The innermost layer of the retina is the nerve fiber layer (NFL) and is composed by the ganglion cell axons, which constitute the optic nerve (ON) fibers that converge to the optic disk. The astrocytes are also distributed in the NFL and in the optic nerve head (ONH). The inner limiting membrane is a basement membrane that forms the interface between the retina and the vitreous

chamber, and it is composed by astrocyte processes and Müller cell endfeet (Dalkara et al., 2009).

The neurosensory retina contains a diversity of cell types. However, three main cell types can be distinguished: neurons, glial cells and vascular cells.

1.1.2. Neurons

The photoreceptor cells are specialized neurons responsible for the transduction of light into electrical signals that can be interpreted by the brain. Apart from the photoreceptors, four major classes of neurons can be found in the retina: horizontal, bipolar, amacrine and retinal ganglion cells (Purves and Williams, 2001). The connection between the photoreceptors and the brain is mediated by these neurons through different pathways. Rods and cones synapse with integrator neurons in the OPL. Nevertheless, the pathway for signal transmission is different for rods and cones. In the cone pathway, the cones synapse with bipolar cells in the OPL and bipolar cells communicate with RGCs in the IPL. The signal transmission though the rods involves connection to RGCs via bipolar and amacrine cells in the IPL (la Cour and Ehinger, 2006). The horizontal cells synapse in the OPL and connect rods and cones with bipolar cells. These neurons always have an inhibitory output (Purves and Williams, 2001). Bipolar cells have their dendrites in the OPL, where they receive signal directly from cones and rods, or via horizontal cells. Their axons extend into the IPL where they synapse with amacrine cells or RGCs. Unlike photoreceptors and horizontal cells that hyperpolarize in response to light, some bipolar cells hyperpolarize and others depolarize. Thus, sending opposite signals to RGCs, that can either increase or decrease the frequency of action potentials – ON and OFF center ganglion cells, respectively (Werner and Chalupa, 2004). The amacrine cells mediate the communication between bipolar cells, other amacrine cells and RGCs in the IPL. The RGCs are the last neurons in the chain. They transmit their signals along their axons (that form the ON) into the visual cortex, superior colliculus and lateral geniculate nucleus. Unlike the other retinal neurons, these cells fire action potentials. The temporal and spatial integration of the inputs from bipolar cells and amacrine cells leads to the final output of the RGCs (Münch, 2010).

1.1.3. Glia

In addition to neurons, the retina also contains four types of glial cells that have support and structural roles, removing cell debris and contributing for retinal homeostasis. Moreover, glial cells also have an important functional role in the modulation of neuronal

physiology (Shaham, 2005). They consist of two main populations: the macroglia, which comprises Müller cells, astrocytes and oligodendrocytes, and the microglia. However, the oligodendrocytes are usually not present on mammals' retina, but they are seen when myelinated RGC axons are present in the NFL, e.g. in chick retina (Rompani and Cepko, 2010).

The Müller cells are the most abundant glial cell type in the retina, comprising 90% of the retinal glia. Also called as the radial glia, their small projections span into the spaces between neurons and contact with retinal blood vessels in the inner blood-retinal barrier (BRB) (Fig. 3) (Runkle and Antonetti, 2011). Under normal conditions, Müller cells regulate extracellular ion concentration and glutamate uptake and provide stability to neural tissue (Bringmann et al., 2006). In response to retinal injury, these cells can proliferate and express inflammatory markers, such as glial fibrillary acidic protein (GFAP) in retinal ischemia-reperfusion injury (Wurm et al., 2011).

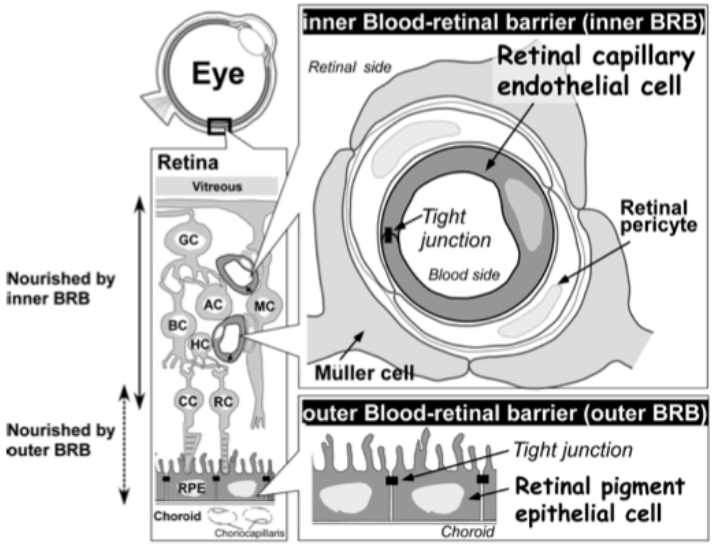

Figure 3. Schematic representation of inner and outer blood-retinal barriers. GC, retinal ganglion cell; AC, amacrine cell; BC, bipolar cell; HC, horizontal cell; MC, Müller cell; RC, rod photoreceptor cell; CC, cone photoreceptor cell; RPE, retinal pigment epithelial cell. Adapted from Tachikawa et al., 2012.

Astrocytes are star-shaped cells that are distributed in the IPL, RGCL and ONH. They are mostly present in the NFL, and their processes contact RGCs, nerve fibers and retinal blood vessels, contributing for the formation of the inner BRB (Runkle and Antonetti, 2011). These cells express GFAP and vimentin under normal conditions, which are commonly used as astrocytic cell markers.

Microglia are the phagocytic cells of the CNS. These cells are involved in the maintenance of retinal homeostasis with their processes in continuous surveillance of their microenvironment. Under stress conditions, microglial cells can proliferate, adopt an

ameboid morphology and migrate from the retinal plexiform layers to the site of injury (Karlstetter et al., 2010). These cells express major histocompatibility complex (MHC) proteins and macrophage antigens, and are considered to contribute to the pathogenesis of several retinal diseases (Langmann, 2007, Karlstetter et al., 2010, Liu et al., 2012, Shima et al., 2012).

1.1.4. Vascular cells

There are two types of retinal blood vessels that nourish the cells of the retina: the central retinal artery capillaries and the choriocapillaries. The first are a network of continuous capillaries that supply the inner retina consisting of endothelial cells with tight junctions, pericytes and glial cells, consisting the inner BRB (Fig. 3). The photoreceptors and RPE are supplied by large choroidal capillaries that are permeable to macromolecules (Fig. 3) (Mitra et al., 2013). The outer BRB consists of an epithelial barrier, the RPE, with tight junctions that control the exchange of substances with the choriocapillaries (Fig. 3). Both components of the BRB control the balanced microenvironment composition of the retina.

1.2. Microglia

Microglia are the resident macrophages of the CNS, accounting for aproximatelly 12% of the cells in the brain (Lawson et al., 1990, Perry and Gordon, 1991). Microglial cells protect and support neuronal functions (Streit, 2002), and are involved in the surveillance and maintenance of the homeostasis of the CNS under both healthy and pathological conditions (Napoli and Neumann, 2009, Ma et al., 2013). Microglia are continuously surveying the surrounding microenvironment with their long protrusions, and they are considered as sensors of the CNS tissues (Ma et al., 2013). Microglia signaling is also involved in synaptic plasticity and neurogenesis (Kettenmann et al., 2011). These cells show specific features that distinguish them from other glial cells. Microglia are also considered the innate immune cells of the CNS, as they are associated with antigen presentation and immunoregulation (Olson and Miller, 2004). In the steady state they express undetectable levels of MHC antigens. However, when participating in host defense against microorganisms and tissue repair, they express MHC antigens (Ransohoff and Perry, 2009).

The origin of microglial cells has been subject of much debate (Cuadros and Navascues, 1998, Chan et al., 2007, Kettenmann et al., 2011, Ginhoux et al., 2013), and a consensus was reached that it does not share the neuro-ectodermal origin of other glial cells

(Priller et al., 2001). It is currently generally accepted that they are of hematopoietic origin. Resident CNS microglia are considered to derive from progenitors that are of myeloid/mesenchymal origin (Rezaie and Male, 2002), that invade the brain parenchyma in early development, and transform into the ramified phenotype (Cuadros and Navascues, 1998). Moreover, in the neonatal and adult brain, microglia are also considered to originate from circulating blood progenitors (monocytes) that cross the blood vessels to the nervous system (Rezaie et al., 2005). These cells can be distinguished from other populations of mononuclear phagocytes that infiltrate the CNS, as they have differentiating electrophysiological properties – they possess inward-rectifying potassium channels (Banati et al., 1991). In the developing retina, microglia progenitors arise from the retinal margin via ciliary body and iris blood vessels, and from the optic disc via retinal vasculature (Diaz-Araya et al., 1995). In the adult retina, the first differentiate into the ramified parenchymal microglia, and the later emerge in the vicinity of blood vessels (perivascular and paravascular microglia) (Provis et al., 1996). During development, the migration of microglia progenitors to the retinal parenchyma is coordinated by the expression of chemokines, including monocyte chemoattractant protein-1 (MCP-1) and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) (Rezaie and Male, 2002). Then, once spread in the parenchyma, these cells are round-shaped (ameboid) with short and broad processes. Their function is the phagocytosis and elimination of cellular debris from apoptotic neurons in the RGCL and INL. Finally, they differentiate into mature, resting and ramified microglia (Cuadros and Navascues, 1998).

The introduction of new transgenic mouse models in which CX3CR1, a specific marker of microglia in the retina (Lee et al., 2008) and in the brain (Nimmerjahn et al., 2005), was replaced with green fluorescent protein (GPF) ($CX3CR1^{+/GFP}$ mice) paved the way for *in vivo* visualization of microglia (Liu et al., 2012). Furthermore, the advances in the optimization of imaging techniques, such as two-photon imaging (Davalos et al., 2005) and confocal scanning laser ophthalmoscopy imaging (Eter et al., 2008), facilitated the *in vivo* study of microglia distribution, morphology and dynamics. In the adult retina, microglia are ramified and show a ordered and pluristratified distribution (Chen et al., 2002), in contrast with broadly distributed brain microglia (Lawson et al., 1990). Ramified retinal microglia reside in the inner and outer plexiform layers (Hume et al., 1983), and occasionally in the ganglion cell layer (Ashwell et al., 1989) (Fig. 4).

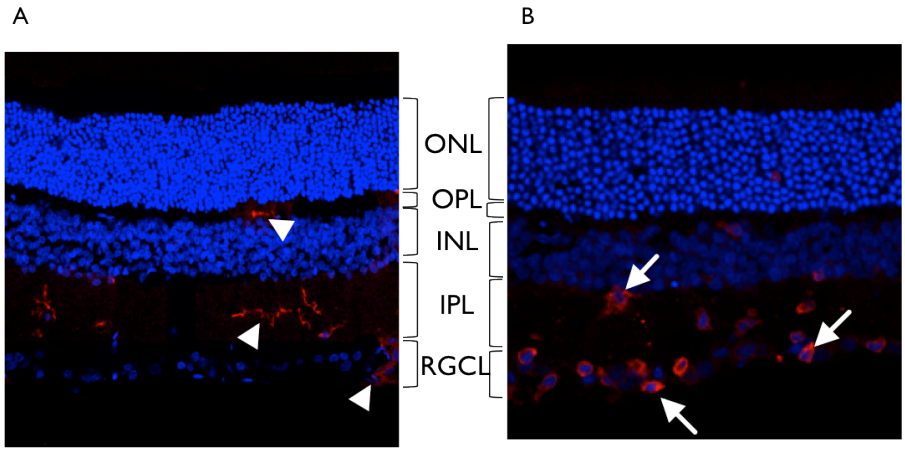

Figure 4. **Sagittal section of the rat retina**, showing that ramified microglia (red) are mainly localized in the plexiform layers and occasionally in the ganglion cell layer (A, arrowheads). When activated, microglia (red) display an ameboid shape and migrate to the injury site after ischemia-reperfusion injury, which affects mainly the inner layers of the retina (B, arrows). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; RGCL, retinal ganglion cell layer.

Unlike previously reported, "resting" microglia are highly dynamic cells, that continuously remodel their processes, act as sensors of their surrounding microenvironment (Raivich, 2005) and secrete supporting neurotrophic factors (Carwile et al., 1998). In the healthy retina, without movement of the soma, they monitor the different retinal layers with their highly motile protrusions to phagocyte cellular debris and clear metabolic byproducts (Nimmerjahn et al., 2005). Stress conditions caused by retinal injury or pathological processes, lead to the activation of microglia from their surveillant state. In response to apoptosis and degeneration of retinal neurons, these cells change their branched and ramified appearance, and adopt ameboid morphology, retracting their processes (Ni et al., 2008). Activated resident microglia then migrate to the site of injury (Fig. 4), proliferate and release pro- and anti-inflammatory factors (D'Orazio and Niederkorn, 1998, Sivakumar et al., 2011, Smith et al., 2012). These substances include tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-10, nitric oxide (NO) and reactive oxygen species (ROS). Nevertheless, damage-associated changes within the retina can also lead to recruitment of blood derived progenitors (Karlstetter et al., 2010). Once activated, microglial cells accumulate in the nuclear layers and participate in the phagocytosis of cellular debris and reparative processes (Chen et al., 2002, Lee et al., 2008). The morphological changes in microglia are accompanied by the expression of several surface markers such as glycoprotein F4/80, the integrin CD11b, the calcium binding protein Iba-1, the leukocyte common antigen CD45, OX-6 (MHC-II) and lysosomal protein ED-1, the rat homolog of human CD68 (Langmann, 2007).

Microglial cells are in continuous communication with surrounding glial cells (astrocytes and Müller cells), vascular elements as well as with neighboring neurons, which

regulate their activation status (Streit, 2002). This communication involves the transmission of signals from glial cells and neurons to microglia through a subset of cell surface molecules on the microglial cell membrane, which include chemokine receptors, cytokine receptors, scavenger receptors and pattern recognition receptors (PRRs) (Hanisch and Kettenmann, 2007). Normal retinal immune regulation include the transmission of diverse signals to microglia, through soluble factors including purine nucleotides (Domercq et al., 2013), the chemokine fractalkine (CX3CL1) (Luhmann et al., 2012), TGF-β and neurotrophic factors (De Simone et al., 2007). Direct contact between microglia and neurons also occur through CD200/CD200 receptor complex. CD200R is exclusively expressed in myeloid cells, including microglial cells. This interaction controls microglial activation and function (Taylor et al., 2011). Microglia are present at the synapses, together with neurons and other glial cells, and are endowed with receptors for several neurotransmitters. Recent data suggests that neurotransmission plays a role in regulating morphology and function of surveillant microglia (Fontainhas et al., 2011).

1.2.1. Microglia and neuroinflammation

Inflammation can be defined as the process, by which the defense cells and molecules of the organism leave the blood stream and enter the injured tissue, in order to destroy the invading pathogen and to initiate the repair of the injured tissue. In the CNS, inflammation can cause neuronal damage and death, originating a cycle that can be self-perpetuated by neurotoxic factors that exacerbate the degeneration of neurons (Ransohoff and Perry, 2009). In the non-damaged CNS the separation from the noxious substances in the peripheral blood stream is afforded by two barriers - by the blood-brain barrier (BBB) in the brain, and by the BRB in the retina. BBB/BRB disruption can serve as an activating signal for local inflammatory cells, but also allows the infiltration of leukocytes from the periphery (Block and Hong, 2005). However, the infiltration of leukocytes not always results in neurotoxicity, envisaging an important role for local glial cells in the inflammatory response to neurodegeneration (Block and Hong, 2005).

In response to different stimuli, microglia develop an early and rapid response, which is defined by the nature of the insult that triggers apoptosis and degeneration of neurons (Schuetz and Thanos, 2004). Although microglia are mononuclear phagocytic system family members, they need to be regarded as CNS glial cells involved in the response to danger signals leading to an acute inflammatory response.

Toll-like receptors (TLRs) belong to the family of pattern-recognition receptors that are activated by the gram-negative bacterial cell wall endotoxin lipopolysaccharide (LPS). TLR4 is considered as the primary LPS receptor (Triantafilou and Triantafilou, 2002), and mediates microglial response to LPS. In addition, TLR4 can also bind LPS in a CD-14 dependent process (Godowski, 2005). Microglia express several surface receptors capable of binding different pathogen-associated molecular patterns, triggering a signaling cascade and eliciting an immune response (Kettenmann et al., 2011). Engagement of TLR provides an important mechanism for the study of microglia interactions with exogenous and endogenous ligands within the CNS. LPS is a widely used endotoxin for the activation of microglia and peripheral immune cells (Wang et al., 2011, Olajide et al., 2013). Although LPS has no direct toxic effect on neurons, it can induce microglia-mediated neurotoxicity (Kim et al., 2000, Block et al., 2007). Activated microglia release a host of neurotoxic factors that are able to induce neuronal cell death, contributing to the initiation and propagation of the neurodegenerative process. As an example, in a model of experimental glaucoma, LPS is described to exacerbate the loss of RGCs (Chiu et al., 2010). Upon activation, the majority of factors released by microglia are pro-inflammatory and neurotoxic. These include pro-inflammatory cytokines such as TNF-α (Sivakumar et al., 2011), IL-1β (Sivakumar et al., 2011) and IL-6 (Sappington and Calkins, 2006) and free radicals including NO (Ferreira et al., 2010) and superoxide radicals (Kanamori et al., 2010) (Fig. 5). LPS can also induce the expression of inducible nitric-oxide synthase (iNOS) (Ferreira et al., 2010) and the production of fatty acid metabolites like eicosanoids (Choi et al., 2009) in microglial cells (Fig. 5). Peroxynitrite, a product of superoxide and NO, is also a major mediator of neurotoxicity induced by LPS (Li et al., 2005) leading to nitrosative stress in the CNS.

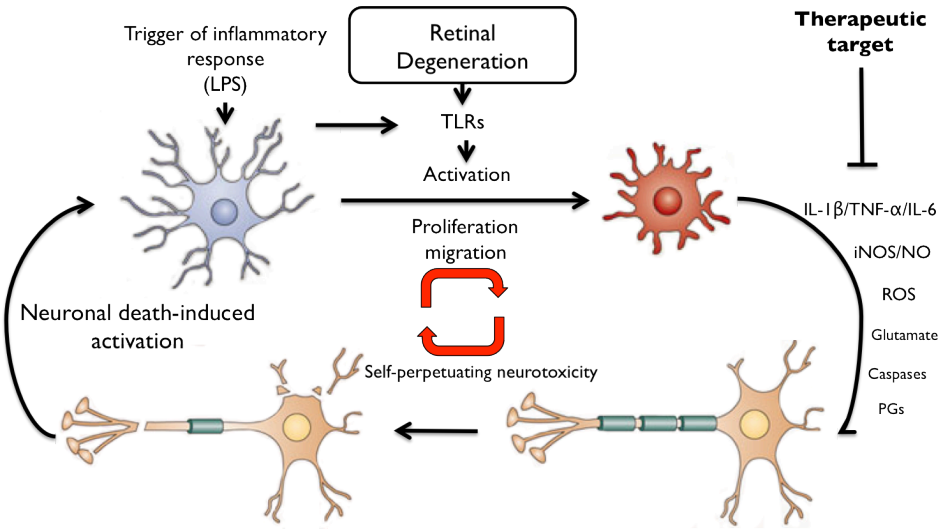

Figure 5. Mechanisms of microglia response to neuroinflammation. First, activation of ramified microglia can be triggered by pro-inflammatory stimuli such as LPS, resulting in the production of pro-inflammatory neurotoxins. In

response to neuronal damage, microglia can become overactivated, which induces toxicity to neighbouring neurons, perpetuating neuronal death. Second, several triggers of retinal degeneration can initiate TLR signaling leading to microglia activation and production of pro-inflammatory neurotoxic factors, thus leading to chronic microglia activation and neuronal apoptosis. Adapted from Perry et al., 2010.

Interestingly, some neurotoxins by acting directly on neurons induce microglia activation that once activated, can remain in this state becoming overactivated. In this way, some neurotoxins induce neuronal damage by a direct action on neurons and indirectly by overactivating microglia (Fig. 5). Furthermore, microglia are also reported to play an important role in neuronal survival in response to injury (Neumann et al., 2006, Polazzi and Monti, 2010) by releasing neurotrophic factors such as brain derived neurotrophic factor (BDNF) (Elkabes et al., 1996), ciliary neurotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) (Carwile et al., 1998), anti-inflammatory cytokines like IL-10 (Seo et al., 2004) and by phagocytosing dead neurons and clearing cell debris (Karlstetter et al., 2010). In addition, stimulation of microglia with several inflammogens leads to activation of the effector caspases in microglia through a protein kinase C (PKC)-dependent pathway (Burguillos et al., 2011), contributing to neurotoxicity. In fact, depending on the type of stimulus and progression of the disease, microglia activation does not always lead to neuron death. Albeit, an unregulated response or overactivation of microglia can result in deleterious consequences (Fig. 5). Fortunately, several endogenous protective molecules have been identified that inhibit microglial overactivation, such as anti-inflammatory cytokines like IL-10 and TGF-β (D'Orazio and Niederkorn, 1998), neuropeptides (Block et al., 2006), cannabinoids (More et al., 2013), glucocorticoids (Glezer and Rivest, 2004) as well as microglia apoptosis (Dragunow et al., 2006).

1.2.2. Microglia and retinal degeneration

Microglia activation is considered to be a hallmark of several neurodegenerative diseases, and it has been linked to the pathology and disease progression of several diseases, including Alzheimer's disease (Lue et al., 2001), Parkinson's disease (Block et al., 2007), multiple sclerosis (Friese and Fugger, 2007) and amyotrophic lateral sclerosis (Turner et al., 2004). Reactive microglia were found to interact with amyloid beta peptide plaques (Lue et al., 2001) activating microglia to release neurotoxic factors (Qin et al., 2002). Additionally, growing evidence also shows a major role of microglia in retinal neurodegenerative diseases. In the context of neurodegeneration, the role of microglia has markedly changed over the years. In the recent years, several reports describe that activated microglia, are the main mediators of retinal neuroinflammation, having a major contribution to this process (Gupta

et al., 2003, Schuetz and Thanos, 2004, Zeng et al., 2008, Kaur et al., 2013). Retinal degeneration triggers TLR signaling, leading to microglia activation, proliferation and migration to the injury site. Activated microglia secrete several bioactive molecules, initially active in tissue repair. Due to the existing cross-talk between microglia and neurons, the damaged or dying neurons additionally stimulate microglia activation, amplifying the neuroinflammatory process in the retina. Chronic microglia activation may lead to exaggerated microglia responses and retinal damage that results in massive neuronal apoptosis a known feature of retinal neurodegenerative diseases (Fig. 5) (Schuetz and Thanos, 2004).

One of the most common retinal degenerative diseases in the world is glaucoma, and by the year 2020 it is estimated that it will globally affect almost 80 million people (Cook and Foster, 2012). Glaucoma is a degenerative optic neuropathy, characterized by RGC death, which contributes to vision impairment and blindness. RGCs are particularly susceptible to different insults, such as retinal ischemia, and undergo apoptosis in several retinal neurodegenerative diseases with particular emphasis to glaucoma (Guo et al., 2005). Current treatments are limited to lowering IOP, but a significant number of patients continue to lose vision despite successful IOP control. Therefore, other mechanisms responsible for RGC degeneration remain unknown. One of the possible mechanisms with a role in glaucomatous optic neuropathy pathology is the insufficient blood perfusion to the ONH, as a result of increased IOP. As the perfusion pressure (arterial blood pressure minus IOP) to the ON depends on the IOP, elevated IOP might cause increased pressure within the retina, especially in the ON. Thus, reduced blood flow in the ON may lead to ischemia (Flammer et al., 2002). Retinal ischemia reperfusion (I/R) injury is described to cause degeneration in the inner retina, including RGCs (Selles-Navarro et al., 1996, Tong et al., 2012). It is described a peak in the number of apoptotic cells in the INL and RGCL after twenty four hours of reperfusion. However, apoptosis of these cells can be detected as soon as six hours after reperfusion (Chen et al., 2003).

Accumulating evidence shows an active role of microglial cells in the pathogenesis and progression of glaucomatous injury. Using a transgenic mouse model of glaucoma it has been demonstrated an early activation of microglia in the central retina and ONH and later redistribution on the retinal periphery (Bosco et al., 2011) and upregulation of genes related with immune response and glial activation (Steele et al., 2006). In a model of ocular hypertension, the presence of microglia on the ONH was detected during the peak of RGC death with increased expression of CD200R (Taylor et al., 2011). Furthermore, Iba-1 and

MHC-II expression in microglial cells is upregulated upon induction of ocular hypertension (Gallego et al., 2012).

Noteworthy, the inflammatory response of microglia can also be neuroprotective depending on the phase of disease progression. For example, using a laser photocoagulation glaucoma model, MCP-1 can induce changes in the activation state of microglial cells, exhibiting a neuroprotective role on the RGC survival with increased levels of insulin-like growth factor-1 (Chiu et al., 2010).

Retinal ischemia is a pathological condition that occurs when the blood supply is reduced to an insufficient level to meet retinal metabolic demands. It is a common cause of visual impairement and blindness (Osborne et al., 2004). Retinal ischemia is often associated with glaucoma, central retinal artery or vein occlusion and diabetic retinopathy. Retinal I/R is a well characterized model to study retinal ischemia. Retinal ischemia is induced by elevating IOP above the systemic arterial blood pressure for a defined period of time, and after that period restoration of blood flow is allowed (Pinar-Sueiro et al., 2013). This results in retinal ischemic damage that is aggravated by the reperfusion-induced damage resulting in functional alterations in retina (Chen and Tang, 2011). As soon as six hours after reperfusion, microglial cells become activated, acquiring an ameboid morphology and expressing ED-1, a marker of microglial/phagocytic cells (Zhang et al., 2005a).

I/R injury involves several mechanisms that result in necrotic and apoptotic cell death. The expression of pro-inflammatory cytokines has been described. Protein and mRNA levels of IL-6 in ischemic retinas are upregulated, with ED-1-positive cells expressing IL-6 present in the inner retinal layers (Sanchez et al., 2003). Additionally, the levels of TNF-α (Berger et al., 2008) and IL-1β (Yoneda et al., 2001) are elevated in the ischemic retinas 12 to 24 hours after ischemia, inducing apoptotic RGC death (Berger et al., 2008, Zhang et al., 2012). However, necrotic RGC death can also be found in ischemic retinas (Shibuki et al., 2000). In another study, using TLR4-deficient mice, it was demonstrated that TLR4 signaling contributes to retinal damage and inflammation triggered by retinal I/R injury (Dvoriantchikova et al., 2010). Altogether, stimulation of TLR4 and elevated levels of TNF-α and IL-1β leads to an increase of the expression and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 after I/R injury (Sanchez et al., 2003). Furthermore, upregulation of iNOS may further contribute to retinal damage after I/R (Cho et al., 2011). At one day after ischemia it was described that the number of microglial cells present in the IPL and RGCL increases, acquire a rounder morphology and express OX-6, a MHC-II marker (Zhang et al., 2005a). Microglia finally recover their ramified morphology at the end of the first week forward (Zhang et al., 2005a), accompanied by decreased retinal

thickness and a dramatic reduction in the number of RGCs (Chen and Tang, 2011). In conclusion, retinal I/R results in activation of microglia, with production of neurotoxic factors that induce the degeneration of RGCs, supporting the hypothesis that microglia contributes to the induction and progression of glaucoma and other retinal neurodegenerative diseases.

1.3. Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid (aa) peptide with five tyrosine (Y) residues in its primary structure, which was first identified and sequenced in the ninety eighties decade (Tatemoto, 1982). Evolutionarily, this peptide is well conserved. NPY synthesis occurs starting from the pre-pro-NPY precursor (97 aa protein) in the endoplasmatic reticulum by a peptide signal sequence. Cleavage of this signal sequence by a signal peptidase generates pro-NPY (69 aa protein), which is further processed by prohormone converting enzymes resulting in NPY(1-39) and C-terminal flanking peptide of NPY. This fragment is finally processed by carboxypeptidase H and peptidylglycine α-amidating monoxygenase to yield the mature amidated 36-aa peptide (Fig. 6) (Walther et al., 2011).

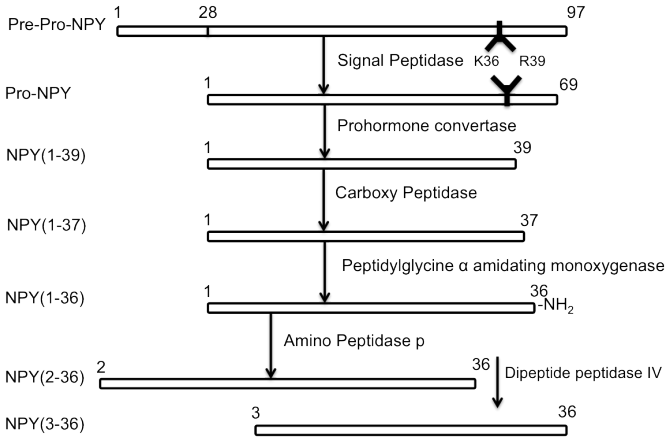

Figure 6. Biosynthesis of Neuropeptide Y. NPY is processed from the 97 amino acids precursor protein pre-pro-NPY, directed to the endoplasmic reticulum by a signal peptide sequence. Adapted from Patel and Patel, 2010.

The NPY family consists of three native ligands: NPY and two gut hormones peptide YY (PYY) and pancreatic polypeptide (PP). Notwithstanding structural differences between these polypeptides, they share a common hairpin-like three-dimensional structure (PP-fold), a sequence containing 36 amino acid residues and an amidated C-terminus (Fig. 7).

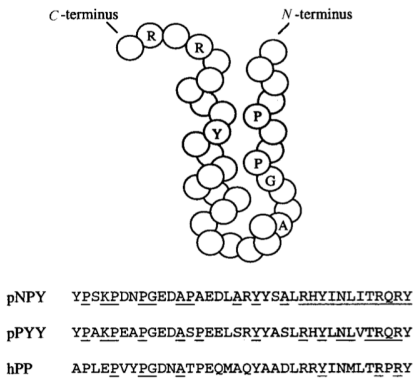

Figure 7. The NPY family native ligands. On the top, it is illustrated the hairpin-like three-dimensional structure (PP-fold). On the bottom, the homologous positions in amino acid positions for each peptide are underlined. Adapted from Cabrele and Beck-Sickinger, 2000.

Moreover, NPY shows a 70% homology with PYY and a 50% homology with PP (Cabrele and Beck-Sickinger, 2000). Pharmacologically, these polypeptides exhibit different properties. NPY can act as a neurotransmitter or a neuromodulator depending on the milieu whereas PYY and PP act as neuroendocrine hormones. PP is mainly found in the endocrine pancreas and it is secreted in the Langerhans islets after food ingestion and promotes appetite suppression (Suzuki et al., 2010). PYY is expressed by entero-endocrine cells of the gut (Lundberg et al., 1982) and it acts both on peripheral and CNS receptors. The predominant form of PYY is released in the circulation (PYY_{3-36}) yielding anorexigenic effects (Pittner et al., 2004).

NPY is present in sympathetic neurons where it is released along with noradrenaline and adenosine triphosphate (Wier et al., 2009). Its effects include vasoconstriction and regulation of blood pressure (Walker et al., 1991), among others. It is described as a potent orexigenic peptide, being synthetized and released by arcuate nucleus neurons (Sousa-Ferreira et al., 2011). NPY is the most abundant peptide in the mammalian brain and is widely distributed in the CNS (Silva et al., 2005), including the retina (Alvaro et al., 2007). It acts as a neurotransmitter in different brain regions and can be neuroprotective under several noxious conditions both in the brain (Silva et al., 2005, Baptista et al., 2012, Goncalves et al., 2012) and the retina (Alvaro et al., 2008b, Santos-Carvalho et al., 2013b). Moreover, NPY is know to promote hippocampal (Decressac et al., 2011) and retinal (Alvaro et al., 2008a) neurogenesis, and to stimulate the production of neurotrophins in the brain (Gelfo et al., 2011). Growing evidence suggests that NPY plays an important role in the immune system and inflammation, particularly in the modulation of microglia/macrophage function (De la Fuente et al., 2001, Ferreira et al., 2010, Ferreira et al., 2012, Goncalves et al., 2012). The presence of NPY-positive fibers in lymphoid organs and in direct contact with immune cells illustrates a role for NPY in the neuroimmune crosstalk (Romano et al., 1991).

The NPY family is a multireceptor system consisting of five receptors, in mammals, which have already been cloned: Y_1R, Y_2R, Y_4R, Y_5R and y_6R. However, only four receptors are functional in humans (hY_1R, hY_2R, hY_4R and hY_5R). The existence of Y_3 receptor is still controversial (Lee and Miller, 1998), and y_6 receptor is only found active in mouse and rabbit (Starback et al., 2000). NPY receptors (NPYRs) belong to the class A (rhodopsin-like) G_i and G_0 protein-coupled receptors. Activation of NPYRs by NPY primarily leads to the inhibition of adenylyl cyclase and finally to decreased cyclic adenosine monophosphate (cAMP) production in the cells (Fig. 8) (Cabrele and Beck-Sickinger, 2000). Furthermore, the activation of the G protein complex can also lead to the modulation of Ca^{2+} and K^+ channels (Brothers and Wahlestedt, 2010). Besides this, depending on cell type, NPYRs can also couple to G_q protein activating phospholipase C and ultimately increasing the levels of inositol 1,4,5-triphosphate (Pedragosa-Badia et al., 2013).

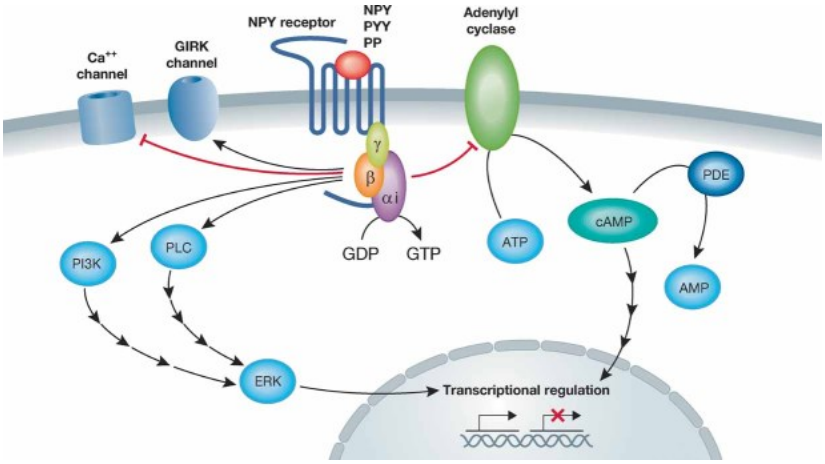

Figure 8. Overview of NPY receptors signal transduction. NPY receptors couple to the G protein signalling cascade, leading to the inhibition of adenylyl cyclase. Furthermore, the activation of the G protein complex can also lead to decreased Ca^{2+} channel activity and enhanced G protein coupled inwardly rectifying potassium (GIRK) currents. Adapted from Brothers and Wahlestedt, 2010.

Unexpectedly, NPYRs family shows low overall sequence similarity (Walther et al., 2011). However, they all bind NPY, but with different affinities. NPY and PYY bind preferably to Y_1R, Y_2R and Y_5R with similar affinities, and PP binds to Y_4R with very high affinity (Cabrele and Beck-Sickinger, 2000). NPY and PYY can also activate Y_4R with minor potency (Cabrele and Beck-Sickinger, 2000). Y_4R is primarily implicated the regulation of food intake and gastrointestinal motility (Berglund et al., 2003, Holzer et al., 2012).

1.3.1. Y_1 Receptor

The Y_1R has 384 aa and it was the first receptor being cloned in the NPY family. This receptor shows high affinity for NPY, PYY and Pro^{34}-substituted NPY/PYY analogs, and low affinities for analogs lacking the N-terminal motif and for PP (Cabrele and Beck-Sickinger, 2000). The C-terminus of NPY is involved in the interaction with this receptor and in its activation (Cabrele and Beck-Sickinger, 2000). This receptor subtype is rapidly internalized upon agonist exposure (Walther et al., 2011). Y_1R mRNA was originally identified in the rat brain (Eva et al., 1990). This receptor subtype is well conserved across all mammalian species (Larhammar et al., 2001). The Y_1R receptor is widely distributed in the CNS. In the brain, it is expressed in the hypothalamus, hippocampus, neocortex, thalamus and amygdala (Caberlotto et al., 1997, Cabrele and Beck-Sickinger, 2000, Wolak et al., 2003). It is also expressed in the retina (Alvaro et al., 2007, Santos-Carvalho et al., 2013a), namely in retinal neurons and retinal glial cells, including microglia (Alvaro et al., 2007, Santos-Carvalho et al., 2013a). Additionally, Y_1R is also found in adipose tissue (Castan et al., 1993), blood vessels (Cabrele and Beck-Sickinger, 2000, Silva et al., 2003a) and adrenal gland (Cavadas et al., 2006, Rosmaninho-Salgado et al., 2007).

This receptor acts postsynaptically, and the presence of Y_1R in blood vessels mediates vasoconstriction (Cabrele and Beck-Sickinger, 2000). Y_1R also plays a role in bone homeostasis (Sousa et al., 2012). The anxiolytic effects of NPY are mediated by this receptor (Lach and de Lima, 2013). In the hippocampus, activation of Y_1R has a neuroprotective role against AMPA and kainate-induced excitotoxicity (Silva et al., 2003b) and mediates NPY-induced neuronal proliferation and differentiation (Decressac et al., 2011). Moreover, activation of Y_1R is involved in the inhibition of intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase in retinal neurons (Alvaro et al., 2009).

In the past years, this receptor subtype has been increasingly described as an important player in the immune system. For the interaction of NPY with the cells of the immune system, these should be equipped with NPY receptors. The expression of Y_1R has been demonstrated in leukocytes, including T, B lymphocytes and antigen-presenting cells, like dendritic cells, macrophages and microglia (De la Fuente et al., 1993, Petitto et al., 1994, Bedoui et al., 2003, Wheway et al., 2005, Ferreira et al., 2010, Santos-Carvalho et al., 2013b). Y_1R activation is involved in the modulation of several immune processes, and mediates several NPY functions in immune cells: suppression of T cell activation and Th1 responses (Bedoui et al., 2003, Wheway et al., 2007), inhibition of phagocytosis by microglial cells (Ferreira et al., 2011), inhibition of cytokine release by macrophages (Straub et al.,

2000, De la Fuente et al., 2001), increase of ROS by macrophages (Dimitrijevic et al., 2005) and NO production by macrophages and microglia (Dimitrijevic et al., 2006, Ferreira et al., 2010).

1.3.2. Y_2 Receptor

The 381 aa Y_2R has high affinity for NPY and PYY, but unlike Y_1R, also shows high affinity for C-terminal fragments [NPY(3-36) to NPY(22-36)] and low affinity for PP (Cabrele and Beck-Sickinger, 2000, Fallmar et al., 2011). This receptor is highly conserved between mammal species. The Y_2R distribution was originally demonstrated in the rat brain (Dumont et al., 1993). It is primarily expressed in several brain regions, including the hippocampus, thalamus, hypothalamus and brain cortex (Gehlert, 1994, Walther et al., 2011). Y_2R is also present in retinal neurons and retinal glial cells (Alvaro et al., 2007, Santos-Carvalho et al., 2013a). Furthermore, the Y_2R is expressed in the peripheral nervous system, in the intestine and blood vessels (Walther et al., 2011).

In contrast to the Y_1R, the Y_2R is not internalized after agonist binding, or it is only to a small extent (Cabrele and Beck-Sickinger, 2000). The Y_2R as a role in angiogenesis (Lee et al., 2003a) and bone formation (Lundberg et al., 2007). This receptor is mainly found pre-synaptically, inhibiting neurotransmitter release (Weiser et al., 2000). Y_2R is involved in the regulation of circadian rhythm (Huhman et al., 1996), learning and memory processing (Redrobe et al., 2004). Additionally, there is evidence that this receptor is involved in epilepsy (El Bahh et al., 2002, Woldbye et al., 2010). The blockade of Y_2R suppresses NPY anti-epileptic effects (El Bahh et al., 2002). The activation of Y_2R also has a neuroprotective role against kainate-induced excitotoxicity in the hippocampus (Silva et al., 2003b), as well as in animal models of cerebral ischemia followed by reperfusion (Smialowska et al., 2009) and Parkinson's disease (Decressac et al., 2012). Furthermore, the protective effect of NPY against methamphetamine-induced toxicity in hippocampal neurons and microglia is mediated by Y_2R (Goncalves et al., 2012). In the retina, Y_2R activation inhibits the increase of $[Ca^{2+}]_i$ concentration in rod bipolar cell terminals (D'Angelo and Brecha, 2004) and has a neuroprotective role against necrotic cell death (Santos-Carvalho et al., 2013b).

In addition, Y_2R is expressed by immune cells and mediates the proadhesive effect of NPY on macrophages (Nave et al., 2004), modulates the production of ROS (Dimitrijevic et al., 2005) and the production of NO induced by LPS (Dimitrijevic et al., 2008). Moreover, this receptor is overexpressed by inflammatory cells upon LPS exposure (Nave et al., 2004).

1.3.3. Y_5 Receptor

The Y_5R represents the most recently cloned receptor on the NPY family of receptors. Two isoforms can be found: 455 aa (long isoform) and 445 aa (short isoform), with similar pharmacological profile (Cabrele and Beck-Sickinger, 2000). The Y_5R is very well conserved among mammalian species (88-90%) (Gehlert, 2004). NPY and PYY are the main ligands for this receptor, however it also shows affinity for Pro^{34}-substituted analogs and lower affinity for PP (Walther et al., 2011). Selective peptides for this receptor subtype include a non-proteinogenic aa residue – aminoisobutiric acid – in their sequence (Cabrele and Beck-Sickinger, 2000).

This receptor subtype is mainly expressed in the CNS, including in the retina (Alvaro et al., 2007), particularly in RGCs and Müller cells (unpublished data).

In the hipothalamus, Y_5R activation has been implicated in food intake (Nguyen et al., 2012) and regulation of the circadian rhythm (Gamble et al., 2005). In the hippocampus, the Y_5 receptors are involved in the modulation of excitatory neurotransmission (Guo et al., 2002) and in the inhibition of seizure-like activity (Woldbye et al., 1997). In addition, activation of this receptor is neuroprotective in hippocampal organotypic cultures (Silva et al., 2003b) and in cortical cultures (Smialowska et al., 2009). Recently, it was described that Y_5R activation prevents apoptotic and necrotic retinal cell death (Santos-Carvalho et al., 2013b). Y_5R activation participates in NPY-induced modulation of inflammatory cells' functions, suppressing the production of ROS and phagocytosis in macrophages (Dimitrijevic et al., 2005) and granulocytes (Dimitrijevic et al., 2006).

1.3.4. Neuropeptide Y system in the retina

NPY and NPYRs are widespread in the CNS, and its presence and function is well documented in several brain regions, and in physiological and pathological conditions (Hokfelt et al., 1998, Ramamoorthy et al., 2011, Malva et al., 2012, Pedragosa-Badia et al., 2013). The retina is often anatomically described as an extension of the brain, illustrating the importance of retinal physiology for a healthy CNS. In the past years, several authors have shed light on the NPY system distribution and function in the retina.

NPY is expressed in the retina of several species (Hokfelt et al., 1998). NPY immunoreactivity (IR) is distributed in different retinal layers and cell types. In the RGCL, NPY-IR was described to be present in the soma and axonal processes of ganglion cells and displaced amacrine cells in several non-mammalian and mammalian species (Bruun et al.,

1986, Hutsler and Chalupa, 1995, Ammar et al., 1998, Sinclair and Nirenberg, 2001), including human RGCs (Straznicky and Hiscock, 1989). NPY gene is also expressed in ganglion cells and amacrine cells in the inner nuclear layer of the mouse retina (Ammar et al., 1998). NPY-IR is further localized in different sublaminas of the IPL, in the INL (amacrine and bipolar cells) and in the OPL (Bruun et al., 1986, Sinclair and Nirenberg, 2001). Additionally, NPY-IR has been detected in rat retinal astrocytes, Müller cells and in microglia, along with mRNA for the Y_1, Y_2, Y_4 and Y_5 receptors in retinal mixed cultures (Alvaro et al., 2007).

NPYRs-IR is specifically present in different cell types of the retina. Recently, using rat retinal cell cultures it has been demonstrated the presence of Y_1 and Y_2 receptors in all types of retinal neurons, such as photoreceptors, bipolar, horizontal, amacrine and retinal ganglion cells. In addition, these receptors were also expressed in astrocytes, Müller and microglial cells in the same retinal cell cultures (Santos-Carvalho et al., 2013a). The mRNA encoding for Y_1, Y_2 and Y_5 receptors has also been detected in the human and bovine RPE (Ammar et al., 1998).

The presence of NPY and NPYRs on the retinal milieu enables NPY and NPY related peptides to exert their physiological functions and uncovers a potential role in retinal degenerative diseases. Indeed, it has been demonstrated that NPY has a neuromodulatory and neuroprotective role in the retina. More specifically, NPY stimulates retinal neural cell proliferation mediated by the activation of Y_1, Y_2 and Y_5 receptors (Alvaro et al., 2008a) and inhibits K^+-evoked $[Ca^{2+}]_i$ increase in retinal neurons through the activation of Y_1, Y_4 and Y_5 receptors (Alvaro et al., 2009). The latter effect on intracellular calcium levels can be seen as a potential mechanism by which NPY can exert neuroprotective effects against retinal neurodegeneration. NPY is released in higher amounts in the injured retina, exerting a proliferative effect on Müller cells via Y_1R activation (Milenkovic et al., 2004). In different studies, it has been demonstrated that NPY has a neuroprotective role against different noxious insults. NPY has a protective effect against 3,4-methylenedioxy-N-methylamphetamine (MDMA)-induced necrosis and apoptosis in rat retinal mixed cultures (Alvaro et al., 2008b).

In the retina, excitotoxicity due to elevated levels of glutamate has been proposed to underlie common retinal degenerative disorders, such as glaucoma and diabetic retinopathy. Excitotoxic damage occurs when ionotropic glutamate receptors, mainly N-methyl-D-aspartate (NMDA) receptors, are overactivated due to excessive levels of glutamate, triggering massive Ca^{2+} influx and activation of pro-apoptotic cascades in neurons, including RGCs (Seki et al., 2010). In a recent study, it was demonstrated that in the retina NPY prevents glutamate-induced necrotic and apoptotic retinal neuronal cell death *in vitro* and *in*

vivo, including RGC degeneration (Santos-Carvalho et al., 2013b). Altogether, these observations suggest that NPY and NPYRs can therefore be envisaged has potential therapeutic targets to treat retinal degenerative diseases.

1.3.5. Neuropeptide Y and neuroinflammation

The immune-privileged status of the CNS is maintained in the brain and in the retina by the BBB and BRB, respectively. Among the constituents of these two barriers, microglia, the resident macrophages of the CNS, are responsible for the maintenance of its integrity. The involvement of these immunocompetent cells has been described in response to injuries and invading pathogens both in the brain (Rogove et al., 2002, Neher et al., 2012) and the retina (Ibrahim et al., 2010, Lorber et al., 2012, Zinkernagel et al., 2013). However, the CNS is not isolated from the immune system. On the contrary, it interacts dynamically with the immune system, and the glial cells actively regulate peripheral immune cells response (Carson et al., 2006).

Growing evidence suggests that NPY plays a pivotal role in the neuroimmune crosstalk. Besides the presence of NPY-positive sympathetic fibers in the lymphoid tissues, NPY is expressed by the immune cells themselves. NPY mRNA has been found in the lymphoid tissue and peripheral blood mononuclear cells, including monocytes (Ericsson et al., 1987). Additionally, NPY is also expressed in microglial cells, including retinal microglia (Alvaro et al., 2007) and in the N9 microglial cell line (Ferreira et al., 2010). The presence of NPYRs has also been demonstrated in several immune cell types. Several reports describe Y_1R expression in immune cells, such as rat lymphocytes, granulocytes and monocytes (Petitto et al., 1994, Bedoui et al., 2002, Nave et al., 2004, Dimitrijevic et al., 2010, Mitic et al., 2011), mice dendritic cells, macrophages, B and T cells (Bedoui et al., 2003, Wheway et al., 2005) and human neutrophils, T cells and granulocytes (Bedoui et al., 2008, Rethnam et al., 2010). Moreover, the expression of Y_2R and Y_5R has also been described in rat granulocytes (Dimitrijevic et al., 2010, Mitic et al., 2011) and human neutrophils (Bedoui et al., 2008). Aditionally, NPY is expressed at sites where immune cells are activated. Interestingly, the expression of NPY in immune cells is increased upon their activation, such as in activated human macrophages, B cells and peripheral blood mononuclear cells (Schwarz et al., 1994) and in mice macrophages (Bedoui et al., 2003). Thus, the expression and release of NPY is inducible in the immune system.

Consistently, NPY effects on both innate and adaptive immune response have been reported, with effects ranging from modulation of phagocytosis (De la Fuente et al., 1993),

production of reactive oxygen and nitrogen species (Dimitrijevic et al., 2006), natural killer cell activity (von Horsten et al., 1998), cytokine production and release (Straub et al., 2000, De la Fuente et al., 2001), chemotaxis and immune cell trafficking (Ahmed et al., 1998, De la Fuente et al., 2001). Noteworthy, signaling through Y_1R on T cells inhibits T cell activation, however on antigen presenting cells enhances antigen uptake and presentation to T cells (Wheway et al., 2005). It is also well described the effects of NPY on macrophage (Fig. 9) and microglia function (Fig. 10).

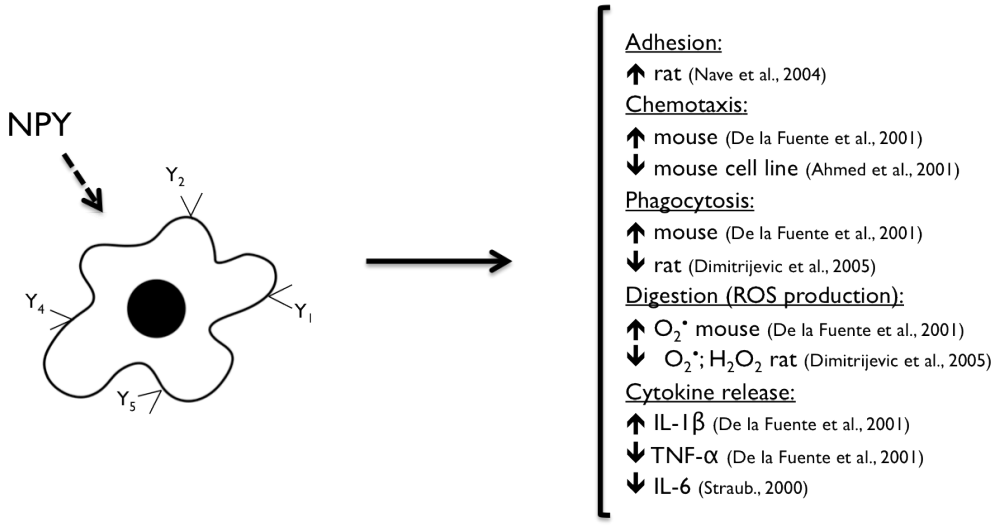

Figure 9. Overview of the effects of NPY on macrophage function. ↑ increase, ↓ decrease. Adapted from De la Fuente and Medina 2005.

NPY enhances adherence of macrophages to the tissue substrate before migrating to the site of inflammation upon LPS exposure through Y_2R activation (Nave et al., 2004). In response to NPY, macrophages migrate towards the focus of infection (chemotaxis) (De la Fuente et al., 2001). Depending on the stimuli, NPY can either stimulate (De la Fuente et al., 2001) or inhibit (Dimitrijevic et al., 2005) phagocytosis, one of the most important and significant functions of macrophages, such as clearance of cell debris. The stimulation of phagocytosis is accomplished by decreasing of cAMP levels and increasing PKC activation (De la Fuente et al., 2001).

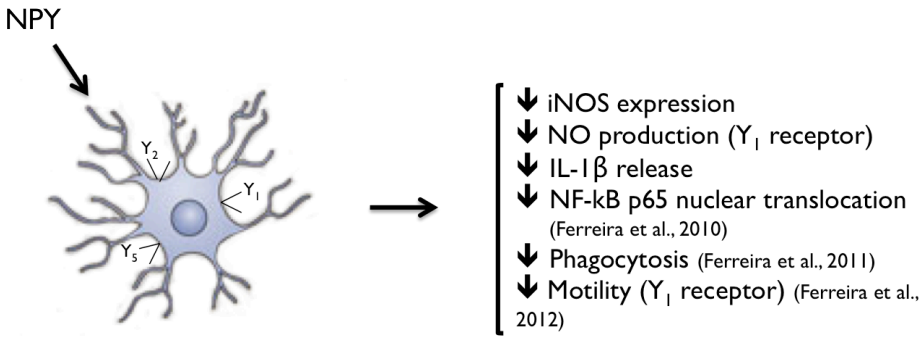

Figure 10. Overview of the effects of NPY on microglia function. ↓ decrease. Adapted from Perry et al., 2010.

In addition, activation of Y_1R by NPY inhibits microglial cell motility and phagocytosis of latex beads, induced by LPS or IL-1β exposure. These effects are mediated by downstream p38 mitogen-activated protein kinase signaling pathway activation (Ferreira et al., 2012) and heat shock protein 27 (Ferreira et al., 2011), respectively.

The production of ROS is crucial for the digestion of phagocytosed material for antigen presentation to lymphocytes. In response to the stimulation with latex beads, NPY can increase superoxide radicals production in macrophages, involving PKC activation (De la Fuente et al., 2001). On the contrary, it has also been reported that upon exposure to zymosan, NPY inhibits superoxide radical release from macrophages (Dimitrijevic et al., 2005). Nevertheless, NPY inhibits IL-6 release from macrophages via Y_1R (Straub et al., 2000) and TNF-α production (De la Fuente et al., 2001), two pro-inflammatory cytokines involved in several inflammatory processes. Regarding IL-1β, another pro-inflammatory cytokine, NPY seems to modulate its levels to keep an homeostatic balance (De la Fuente et al., 2001). More recently, it has been described that NPY inhibits IL-1β release from microglial cells upon LPS activation, inhibiting iNOS and consequently NO production. These effects were exclusively mediated through Y_1R activation and inhibition of nuclear translocation of NF-κB (Ferreira et al., 2010). These data suggest that Y_1R activation may inhibit microglia activation.

NPY can be described has a fundamental player in the crosstalk between neurons and immune cells, evidencing a potential autocrine role in regulating microglia cell functions directly, or in a paracrine fashion, being released by neurons, and in this way modulating microglia overactivation, and ultimately inhibiting the neuroinflammatory response.

MATERIALS AND METHODS

2. Materials and Methods

2.1. Animals

Adult male Wistar rats (250-300 g of bodyweight; Charles River, France) were housed in a temperature- and humidity-controlled environment and were provided with standard rodent diet and water *ad libitum* while kept on a 12-h light/12-h dark cycle. All procedures involving the animals were in agreement to The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Culture of retinal explants

Retinas of 8-9 weeks old male Wistar rats were dissected in Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS in mM: 138 NaCl, 5.3 KCl, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 D-Glucose; pH 7.2) and flat-mounted onto 30-mm diameter culture plate inserts with a 0.4 μm pore size (Millicell, Millipore, USA) with the retinal ganglion cell layer side facing upward. The explants were cultured in six-well plates containing Dulbecco's modified Eagle medium:Nutrient Mixture F-12 (DMEM/F-12) media with GlutaMAX I (Life Technologies, USA) supplemented with heat-inactivated 10% Fetal Bovine Serum (FBS; Life Technologies, USA), and 0.1% gentamicin (Life Technologies, USA), and maintained for four days *in vitro* (DIV) in a humidified incubator at 37°C and 5% CO_2. Culture medium was replaced with fresh media at DIV1 and DIV2.

2.3. Primary cultures of rat retinal neural cells

Primary cell cultures were prepared from the retinas of 3-4 days old Wistar rats as previously described (Santiago et al., 2006). Briefly, rats were euthanized by decapitation, the eyes enucleated and the retinas dissected in ice cold sterile HBSS (pH 7.2). Retinas were incubated for 12 min at 37°C in HBSS containing 0.1% trypsin (w/v; Gibco, USA). Then, cells were pelleted by centrifugation, and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, heat-inactivated 10% FBS, 100 U/ml penicillin (Life Technologies, USA) and 100 mg/ml streptomycin (Life Technologies, USA). The cells were plated at a density of $2.0x10^6$ cells/cm^2, on 0.1 mg/ml poly-D-lysine

(Sigma-Aldrich, USA) coated glass coverslips, and cultured for seven days *in vitro* at 37°C in a humidified atmosphere of 5% CO_2.

2.4. Primary cultures of purified rat retinal microglial cells

Microglial cell cultures were prepared as previously described (Fleisher-Berkovich et al., 2010), with some modifications, as follows. A mixed retinal cell culture was obtained from the retinas of 7-9 days old Wistar rats, as described above. The cells were plated at a density of $1.5x10^6$ cells/cm^2 in 0.1 mg/ml poly-D-lysine coated T75-culture flasks, and cultured for three weeks at 37°C in a humidified atmosphere of 5% CO_2 in DMEM/F-12 with GlutaMAX I, supplemented with heat-inactivated 10% FBS, 0.1% gentamicin and 2 ng/ml macrophage colony stimulating factor (Peprotech, UK). Culture media was fully replaced twice a week.

Microglial cells were obtained from the mixed primary culture by shaking. The culture flasks were placed in an orbital shaker, at 200 rpm for 120 min, at 37°C under a humidified atmosphere of 5% CO_2. Then, cells were pelleted by centrifugation and resuspended in DMEM/F-12 with GlutaMAX I, supplemented with 0.1% gentamicin. Cells were plated at a density of $1.3x10^6$ cells/cm^2 in 0.1 mg/ml poly-D-lysine coated glass coverslips and maintained at 37°C under a humidified atmosphere of 5% CO_2, for three days *in vitro*.

2.5. Drug exposure

NPY (1 µM), [Leu^{31}, Pro^{34}]-NPY ($Y_{1,5}$ receptor agonist; 1 µM; LP-NPY), NPY_{13-36} (Y_2 receptor agonist; 300 nM), and [hPP1-17, Ala^{31},Aib^{32}]-NPY (Y_5 receptor agonist; 1 µM) were obtained from Bachem, Switzerland. BIBP3226 (Y_1 receptor antagonist; 1 µM), BIIE0246 (Y_2 receptor antagonist; 1 µM) and L-152,804 (Y_5 receptor antagonist; 1 µM) were obtained from Tocris, UK.

Cultures were pre-treated with NPY or NPY receptor agonists 1 h before LPS incubation. When present, NPY receptor antagonists were added 30 min before the treatment with NPY or NPY receptor agonists.

LPS was added to the cultures for 24 h, at DIV6 in primary cultures of rat retinal neural cells (1 µg/ml; Sigma-Aldrich, USA), at DIV2 in cultures of purified microglial cells (1 µg/ml), and at DIV3 in cultured retinal explants (3 µg/ml).

2.6. Retinal ischemia-reperfusion

Wistar rats were anesthetized by isoflurane inhalation using a gas anesthetizing system (VetEquip, USA). Then, oxybuprocaine (4 mg/ml; Laboratórios Edol, Portugal) anesthetic was applied topically to the eyes and the pupils were dilated with tropicamide (10 mg/ml; Laboratórios Edol, Portugal). Both eyes were injected intravitreally with 5 μl of NPY (10 μg; Bachem, Switzerland), 5 μl of LP-NPY (10 μg; Bachem, Switzerland) or with 5 μl of sterile saline solution (0.9% sodium chloride; Fresenius Kabi, Portugal) 2 h before ischemia. The anterior chamber of the left eye of the animal was cannulated with a 30-gauge needle connected to a reservoir infusing a sterile saline solution. Retinal ischemia was induced by increasing the intraocular pressure (IOP) to approximately 90 mmHg (TonoLab, Icare, Finland) for 60 min by lifting the reservoir, as previously described (Lee et al., 2012). The contralateral eye of each animal served as non-ischemic control. Retinal ischemia was confirmed by the whitening of the anterior segment of the eye and the loss of the red reflex of the eye fundus, due to interrupted blood flow. After 60 min, the needle was withdrawn, and IOP was normalized. Reperfusion was confirmed by the reappearance of retinal blood flow. Fusidic acid (10 mg/g; Leo Pharmaceutical, Denmark) ointment was applied in the conjunctival sac at the end of the experiment. The animals were allowed to recover for 8 h or 24 h before sacrifice.

2.7. Frozen retinal sections

Rats were deeply anesthetized (75 mg/kg ketamine, and 10 mg/kg xylazine) and transcardially perfused with phosphate buffered saline (PBS; pH 7.4), followed by 4% (w/v) paraformaldehyde (PFA) in PBS. The eyes were enucleated, washed in PBS and then transferred to PFA for 1 h. The cornea and lens were removed and the eye cup was further fixed for 1 h in PFA. After washing in PBS, the tissue was cryopreserved by placing the eye cup in 15% (w/v) sucrose in PBS for 1 h followed by 30% (w/v) sucrose in PBS overnight at 4°C. The eye cup was embedded in tissue-freezing medium (OCT; Shandon, USA), the frozen blocks were cut in a cryostat into 10 μm sections thickness and the cryosections were then collected on SuperFrost Plus glass slides (Menzel-Glaser, Germany). Glass slides were dried overnight and stored at -20°C.

2.8. Immunofluorescence labelling

2.8.1. Cultured retinal explants

Explants were fixed in ice-cold absolute ethanol at 4°C. After washing with PBS, the explants were incubated in 3% bovine serum albumin (BSA), 10% normal goat serum (NGS) and 0.1% Triton X-100 for 1 h at room temperature (RT). Explants were then incubated with primary antibodies (Table 1) for 48 h at 4°C. After washing, they were incubated overnight at 4°C with the corresponding secondary antibody in blocking solution (Table 1). The nuclei were stained with DAPI (1:1,000). The explants were flat-mounted on slides and coverslipped using Glycergel mounting medium (Dako, Denmark) and visualized in a laser scanning confocal microscope (Zeiss LSM 710, Germany).

2.8.2. Primary cultures

Cells were fixed in PFA, washed with PBS and placed in 1% Triton X-100 for 5 min at RT. Unspecific binding was prevented by incubating cells in a 3% BSA and 0.2% Tween 20 blocking solution for 1 h at RT. Cells were incubated with primary antibodies (Table 1) in blocking solution for 90 min at RT. After washing, they were incubated with the corresponding secondary antibody in blocking solution for 1 h at RT (Table 1). The nuclei were stained with DAPI (1:2,000; Life Technologies, USA). The coverlips were mounted on glass slides using Glycergel mounting medium (Dako, Denmark) and visualized in a laser scanning confocal microscope (Zeiss LSM 710, Germany).

2.8.3. Frozen retinal sections

Retinal sections were placed overnight at RT. After fixing with ice-cold acetone for 10 min at -20°C, the sections were hydrated in PBS until OCT was removed. Sections were permeabilized in 0.25% Triton X-100 for 30 min at RT, and blocked in 1% BSA and 10% NGS solution for 30 min in a humidified atmosphere at RT. Sections were then placed overnight at 4°C in a humidified atmosphere with primary antibodies (see table 1) in 1% BSA solution. After washing, they were incubated with the corresponding secondary antibody in 1% BSA solution for 1 h at RT (Table 1). The nuclei were stained with DAPI (1:2,000). The sections were coverslipped using Glycergel mounting medium (Dako, Denmark) and visualized in a laser scanning confocal microscope (Zeiss LSM 710, Germany).

2.9. Quantitative image analysis of immunohistochemical staining

2.9.1. Cultured retinal explants

2.9.1.1. iNOS immunoreactivity

For the quantification of iNOS immunoreactivity in CD11b-positve cells, fluorescence images were taken using a laser scanning confocal microscope (Zeiss LSM 710, Germany) under 40x magnification comprising the four retinal quadrants in each retina. Twelve images per explant were randomly acquired (3 images per retinal quadrant), and densitometric analysis for the iNOS immunofluorescence in CD11b-positve cells was performed using the public domain ImageJ program (http://rsb.info.nih.gov/ij/). The results are expressed in arbitraey units (AU) as iNOS immunoreactivity in CD11b-positive cells/mm^2. Data are presented as mean ± SEM of 5-6 explants.

2.9.1.2. Analysis of microglia morphology

To determine differences in microglia morphology, we used automated features of ImageJ to analyze CD11b-positive cells in laser scanning confocal microscope images (Zeiss LSM 710, Germany) under 40x magnification comprising the four retinal quadrants in each retina. Twelve images per explant were randomly acquired (3 images per retinal quadrant). As previously described (Kurpius et al., 2006), an arbitrary (but uniformly applied) threshold was set to delineate microglial cells. Then, the particle measurement feature in ImageJ was used to automatically measure the 2D area, perimeter, circularity, and Feret's diameter of single microglial cells. Circularity of microglia was calculated using the formula: circularity = 4π(area/perimeter^2). A circularity value of 1.0 indicates a perfectly circular cell, and values near zero indicate ramified cells. Feret's maximum diameter, a measure of cell length, is the greatest distance between any two points along the cell perimeter.

2.9.2. Frozen Retinal Sections

2.9.2.1. ED-1 and OX-6 immunoreactivity

ED-1 and OX-6 immunoreactivity in Iba-1-positive cells was quantified, as previously described (Zhang et al., 2005a). Fluorescence images were taken using a laser scanning confocal microscope (Zeiss LSM 710, Germany) under 40x magnification comprising eight non-consecutive transverse retinal sections. Ten images per section were randomly acquired, and the number of microglial cells (Iba-1-positive) immunoreactive for ED-1 and OX-6 was counted by two independent observers in a blind way. Data was presented as the percentage of ED-1- and OX-6-positive microglial cells/mm^2 of retina.

2.9.2.2. Analysis of microglia morphology

To determine differences in microglia morphology, Iba-1-positive cells were analysed in retinal sections, and classified as ramified or ameboid by two independent observers in a blind way. Ten images per section were randomly acquired under 40x magnification in a laser scanning confocal microscope (Zeiss LSM 710, Germany), covering eight non-consecutive transverse retinal sections. Data was presented as the percentage of ameboid microglial cells/mm^2 of retina.

2.10. Evaluation of reactive oxygen species (ROS) production

2.10.1. Cultured retinal explants

Explants were incubated with dihydroethidium probe (DHE, Sigma-Aldrich, 10 μM) for 1 h at 37°C in fresh culture media. Then, the explants were rinsed twice in warm PBS and fixed in ice-cold absolute ethanol at 4°C, and labelled with anti-CD11b antibody.
Fluorescence images were taken using a laser scanning confocal microscope (Zeiss LSM 710, Germany) under 40x magnification comprising the four retinal quadrants in each retina. Twelve images per explant were randomly acquired (3 images per retinal quadrant), and densitometric analysis for the DHE fluorescence in CD11b-positve cells was performed using ImageJ. The results are expressed in AU as DHE fluorescence in CD11b-positive cells/mm^2, and data are presented as mean ± SEM of 6-7 explants.

2.10.2. Primary purified microglia cultures

Cells were incubated for 30 min at 37°C with DHE (5 μM) in fresh culture media. Then, cells were washed twice in warm PBS and fixed in PFA. The nuclei were stained with DAPI (1:2,000; Life Technologies, USA). The coverlips were mounted on glass slides using Glycergel mounting medium (Dako, Denmark) and visualized in a laser scanning confocal microscope (Zeiss LSM 710, Germany). The DHE fluorescence was quantified in microglial cells. The results are expressed in percentage of DHE fluorescence relatively to the control condition, and data are presented as mean ± SEM of 1-3 retinal microglia cultures.

2.11. RNA extraction and cDNA synthesis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, one retina or retinal explant was mechanically disrupted using a lysis buffer and subsequently homogenized in a QIAshredder homogenizer. Consequently, the sample was transferred to an RNeasy spin column, to yield a RNA-enriched solution. RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). First strand cDNA synthesis was performed using random primers, 0.5 μg total RNA and SuperScript II Reverse Transcriptase (Life Technologies, USA) according to the manufacturer's instructions.

Additionally, genomic DNA contamination was evaluated using a conventional polymerase chain reaction (PCR) for β-actin using intron-spanning primers. Briefly, cDNA (2 μl) was subjected to a 35-cycle PCR amplification using 2x MyTaq Red Mix (Bioline, UK), 200 nM of forward (GCTCCTCCTGAGCGCAAG) and reverse (CATCTGCTGGAAGGTGGACA) primers. PCR products were visualized after electrophoresis on 1.5% (w/v) agarose gels containing 0.005% (v/v) EtBr in Tris-Acetate-EDTA buffer (TAE: 40 mM Tris-Acetate and 1 mM Na_2EDTA). A single band of the anticipated exon-size was found in all samples, demonstrating the absence of genomic contamination. Nontemplate and nonamplicon controls were subjected to PCR amplification, but they never yielded PCR products (data not shown).

2.12. Primer design

Primers for quantitative real time polymerase chain reaction (qRT-PCR) were designed using the Beacon Designer 6 software (PREMIER Biosoft International, USA) for the amplification of gene fragments between 70-110 bp in length and an annealing temperature

(Ta) between 55-60°C. When possible an intron-spanning primer was chosen in order to eliminate amplification of genomic DNA in the cDNA samples. Amplification efficiency of target and reference genes was evaluated using a cDNA ten-fold dilution series and plotting threshold cycle (Ct) values against cDNA dilution (data not shown). Furthermore, at the end of the PCR run, the temperature of the sample was ramped from 60°C to 95°C while continuously collecting fluorescence data, enabling the construction of a dissociation curve. The curves of the melting profiles showed a single product and did not reveal accumulation of primer dimmers, as indicated by a single peak. Primers with amplification efficiency outside of 90-110% range or primer pairs generating multiple peaks were discarded. Final primer sequences and amplicon lengths are shown in Table 2.

2.13. Quantitative real time polymerase chain reaction

qRT-PCR was performed using 20 μl total reaction volume containing 10 μl 2x iTaqTM SYBR® Green Supermix with ROX (BioRad, USA), 200 nM of forward and reverse primers and 2 μl of 1:2 diluted cDNA in a StepOne Plus system (Life Technologies, USA). An initial step of 95°C for 10 min was used to activate the Taq polymerase. PCR cycling conditions were: denaturation at 95°C for 15 s, annealing at primer Ta for 45 s, and elongation at 72°C for 30 s, for 40 cycles. Furthermore, at the end of the PCR a melting curve analysis was performed to evaluate unspecific products and primer-dimer formation. Three technical replicates for each biological sample per group were performed. A non-template control was included for each transcript. Ct values were obtained during the exponential amplification phase using automatic threshold option in StepOne Software (Life Technologies, USA).

2.13.1. qRT-PCR data analysis

Reference gene expression stability between different groups was evaluated using the NormFinder analysis algorithm for Microsoft Excel (Andersen et al., 2004), which identified *Hprt* as the most stable gene in cultured retinal explants (stability value: 0.002), and in ischemia-reperfusion samples (stability value: 0.007). *Hprt* gene was selected as our reference gene for normalization of gene expression in all groups. Relative gene expression data was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where $\Delta\Delta Ct$ = (Ct gene of interest-Ct reference gene)$_{\text{Analyzed Group}}$ - (Ct gene of interest-Ct reference gene)$_{\text{Control Group}}$.

The data analysis was based on 4-12 independent biological samples per group, for cultured retinal explants. Moreover, retinal I/R samples from 6 animals per treatment group were used. The results represent $2^{-\Delta\Delta Ct}$ values and were expressed as the mean ± SEM.

2.14. Enzyme-linked immunosorbent assay

2.14.1. Sample preparation

2.14.1.1. Cultured retinal explants

The production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, in retinal explants was determined by an enzyme-linked immunosorbent assay (ELISA) in the culture medium, after centrifugation at 10,000 g for 5 min.

2.14.1.2. Ischemia-reperfusion

The production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, in the retina was determined by ELISA. The rats were sacrificed after 8 or 24 h reperfusion and retinas 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) containing a protease inhibitor. Samples were centrifuged at 10,000 g for 5 min and the supernatants were diluted three times with lysis buffer. Protein concentrations were determined using BCA Protein Assay Kit (Pierce, USA).

2.14.2. Assay

2.14.2.1. Cultured retinal explants

Samples were used according to the manufacturer's instructions (Peprotech, UK). The absorbance at 405 nm was determined with wavelength correction set at 650 nm using a multimode microplate reader (Synergy HT, Biotek, USA).

The concentrations of TNF-α, IL-1β and IL-6 in the culture medium were calculated according to the standard curve using the recombinant cytokines provided with the ELISA kits.

2.14.2.2. Ischemia-reperfusion

Samples were used according to the manufacturer's instructions (Peprotech, UK). The absorbance at 405 nm was determined with wavelength correction set at 650 nm using a multimode microplate reader (Synergy HT, Biotek, USA).

The concentrations of TNF-α, IL-1β and IL-6 in the retina were calculated according to the standard curve using the recombinant cytokines provided with the ELISA kits, and normalized against the total amount of protein of the samples.

2.15. Statistical analysis

The results are expressed as the mean ± SEM. Data were analyzed by the one-way ANOVA test followed by Bonferroni's multiple comparison test to determine differences between groups or by the unpaired Student's t-test with Welch's correction (IBM SPSS Statistics, USA). Differences were considered statistically significant when the $p<0.05$.

Table 1. Antibodies used in immunofluorescence labeling.

Antibody	Sample	Dilution used	Supplier	Species
Primary antibodies				
Anti-CD11b	Primary culture Explant culture Retinal section	1:100 1:250 1:100	AbD Serotec, Germany	Mouse
Anti-iNOS	Primary culture Explant culture	1:100 1:150	BD Biosciences, UK	Rabbit
Anti-Iba-1	Retinal section	1:1,000	Wako Chemicals, Germany	Rabbit
Anti-ED-1	Retinal section	1:500	AbD Serotec, Germany	Mouse
Anti-OX-6	Retinal section	1:200	AbD Serotec, Germany	Mouse
Anti-NPY	Primary culture Explant culture Retinal section	1:1,000 1:1,500 1:1,000	Sigma Aldrich, USA	Rabbit
Anti-NPY_1R	Primary culture Explant culture Retinal section	1:500 1:1,500 1:500	AbD Serotec, Germany	Sheep
Anti-NPY_2R	Primary culture Explant culture Retinal section	1:500 1:250 1:2,000	Alomone Labs, Israel	Rabbit
Anti-NPY_5R	Primary culture Explant culture Retinal section	1:250 1:100 1:200	Alomone Labs, Israel	Rabbit
Secondary antibodies				
Alexa Fluor® 568 anti-mouse IgG		1:200	Life Technologies, USA	Goat
Alexa Fluor® 488 anti-rabbit IgG		1:200	Life Technologies, USA	Goat
Alexa Fluor® 488 anti-sheep IgG		1:200	Life Technologies, USA	Donkey
Alexa Fluor® 488 anti-mouse IgG		1:200	Life Technologies, USA	Goat

CD11b, cluster of differentiation molecule 11B (integrin alpha M); iNOS, nitric oxide synthase type 2, inducible; NPY, neuropeptide Y; NPY_1R, neuropeptide Y receptor Y_1; NPY_2R, neuropeptide Y receptor Y_2; NPY_5R, neuropeptide Y receptor Y_5.

Table 2. Primers used for qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperatures (°C)	Amplicon size (bp)
Reference genes				
Gapdh	GACTTCAACAGCAACTCC	GCCATATTCATTGTCATACCA	58	105
Hprt	ATGGGAGGCCATCACATTGT	ATGTAATCCAGCAGGTCAGCAA	60	77
Ywhaz	CAAGCATACCAAGAAGCATTTGA	GGGCCAGACCCAGTCTGA	60	76
Target genes				
Tnf-α	CCCAATCTGTGTCCTTCT	TTCTGAGCATCGTAGTTGT	60	90
Il-1β	ATAGAAGTCAAGACCAAAGTG	GACCATTGCTGTTTCCTAG	60	109
Il-6	GGAGAAGTTAGAGTCACAGA	GCCGAGTAGACCTCATAG	60	104
iNOS	AGAGACAGAAGTGCGATC	AGAGATTCAGTAGTCCACAATA	58	96
CD11b	AAGGTCATACAGCATCAGT	GTTGATCTGGACAGGGAT	60	90
Npy	TATCCCTGCTCGTGTGTT	AGCGGAGTAGTATCTGGC	55	107
Npy1r	GGTTGCCGTGATTACTTG	GACAGACAGACAGACACA	58	110
Npy2r	TCTGGGCATCATATCTTTCT	TTCGCTGATGGTAATGGT	55	95
Npy5r	GCATGATGTCCTGTTGTC	TGTGTAGGCAGTGGATAAG	55	93

Gapdh, glyceraldehyde-3- phosphate dehydrogenase; *Hprt*, human hypoxanthine phosphoribosyltransferase; *Ywhaz*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; *Tnf-α*, tumor necrosis factor alpha; *Il-1β*, interleukin 1 beta; *Il-6*, interleukin 6; *iNOS*, nitric oxide synthase 2, inducible; *CD11b*, cluster of differentiation molecule 11B (integrin alpha M); *Npy*, neuropeptide Y; *Npy1r,* neuropeptide Y receptor Y_1; *Npy2r*, neuropeptide Y receptor Y_2; *Npy4r*, neuropeptide Y receptor Y_4; *Npy5r*, neuropeptide Y receptor Y_5.

RESULTS

3. Results

In this work, we aimed evaluating whether the modulation of the NPY system could prevent pro-inflammatory processes in the retina, particularly regulating microglia activation, giving particular attention to Y_1R. For this purpose, we used *in vitro* models, primarily cultured retinal explants, and then confirming the results in more simple retinal culture preparations. We also used a retinal ischemia-reperfusion injury animal model.

3.1. NPY and Y_1R, Y_2R and Y_5R are expressed in retinal microglial cells

It has been previously demonstrated that NPY and Y_1R, Y_2R and Y_5R mRNAs were detected in the N9 microglial cell line (Ferreira et al., 2010). NPY immunoreactivity is detected in retinal microglial cells in a primary culture of purified retinal microglial cells (Alvaro et al., 2007). Recently, it was described that Y_1R and Y_2R are expressed in microglial cells in primary retinal mixed cell cultures (Santos-Carvalho et al., 2013a). However, to date, it is unknown whether the activation of NPY receptors is able to modulate retinal microglia reactivity, particularly in more complex systems, such as cultured retinal explants.

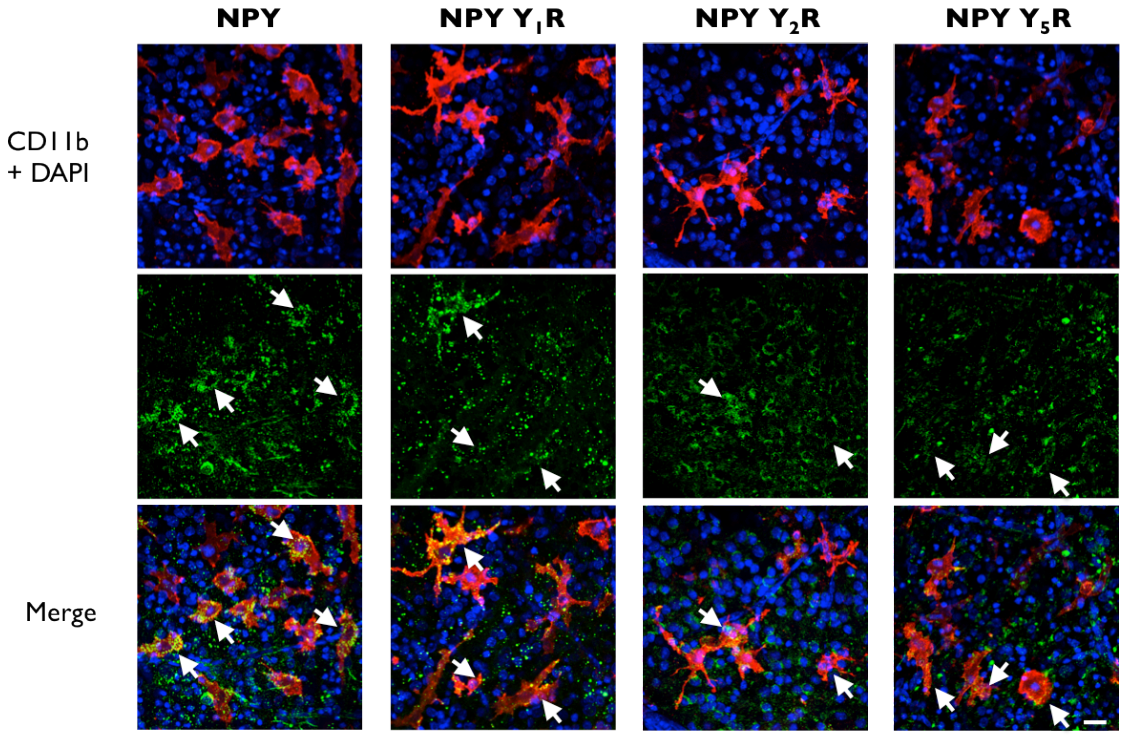

Figure 11. Retinal microglial cells express NPY, Y_1R, Y_2R and Y_5R in cultured retinal explants. Retinal explants were cultured for 4 days. Immunohistochemistry was performed using antibodies against CD11b (red) and NPY, Y_1R, Y_2R and Y_5R (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

The expression of NPY and Y_1R, Y_2R and Y_5R was assessed by immunohistochemistry. In cultured retinal explants, cells were labeled with antibodies that recognize NPY and Y_1R, Y_2R and Y_5R, and microglial cells were labeled with an antibody that recognizes CD11b, an integrin family member that is expressed on the surface of microglial cells (Figs. 11, 12 and 13). NPY and Y_1R, Y_2R and Y_5R immunoreactivity (IR) could be found in CD11b-positive microglial cells in cultured retinal explants (Fig. 11).

NPY and Y_1R, Y_2R and Y_5R expression in CD11b-positive retinal microglial cells was further demonstrated in primary mixed cultures of rat retinal neural cells (Fig. 12) and in primary cultures of purified rat retinal microglial cells (Fig. 13). The primary cultures of retinal neural cells contain retinal neurons and astrocytes, Müller cells and microglial cells.

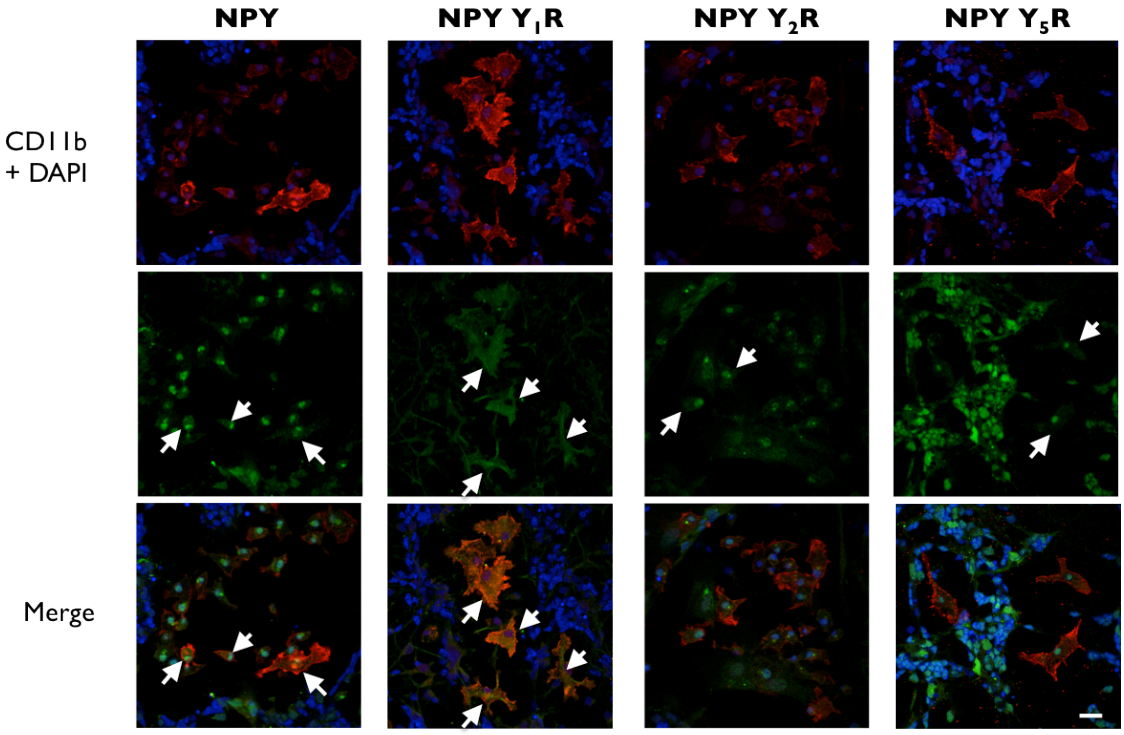

Figure 12. Retinal microglial cells express NPY, Y_1R, Y_2R and Y_5R in primary cultures of rat retinal neural cells. Primary rat retinal neural cells were cultured for 7 days. Immunohistochemistry was performed using antibodies against CD11b (red) and NPY, Y_1R, Y_2R and Y_5R (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

Cultures of purified microglial cells were obtained from primary cultures of retinal neural cells and have a purity of 93±2.3% of microglial cells.

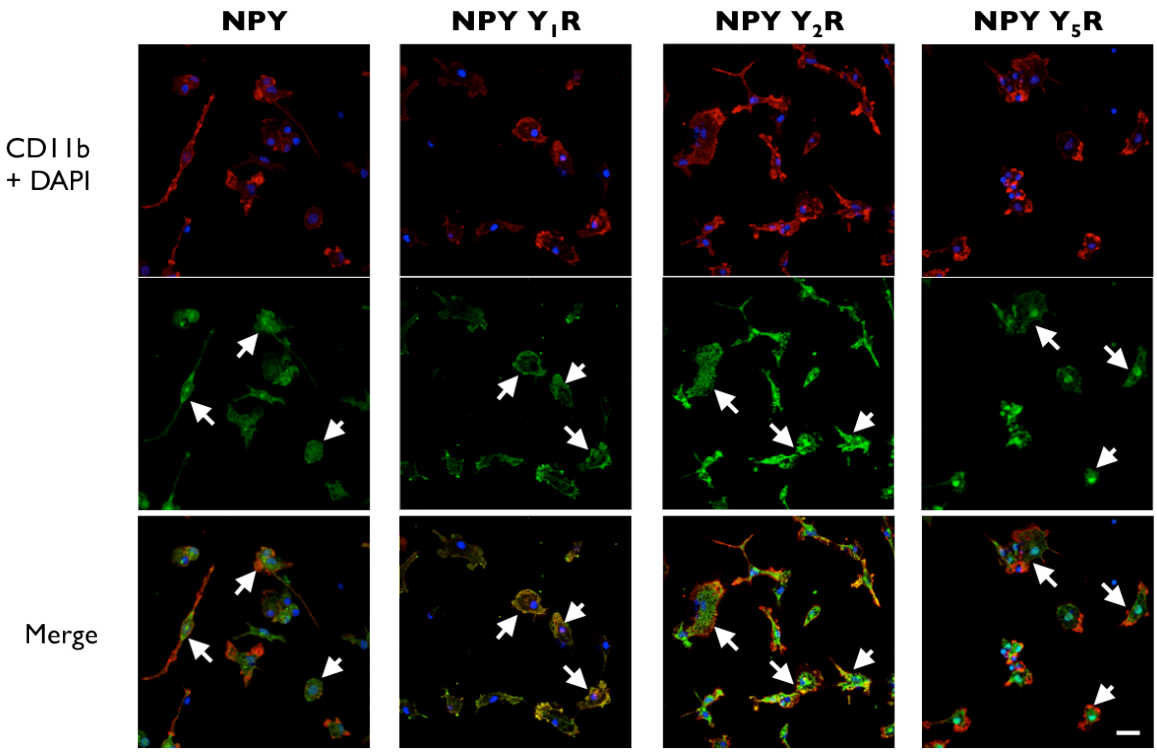

Figure 13. Purified retinal microglial cells express NPY, Y_1R, Y_2R and Y_5R. Primary purified rat retinal microglial cells were cultured for 3 days. Immunohistochemistry was performed using antibodies against CD11b (red) and NPY, Y_1R, Y_2R and Y_5R (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

3.2. NPY and Y_1R, Y_2R and Y_5R mRNAs are expressed in cultured retinal explants. LPS exposure decreases NPY and increases Y_1R mRNA expression

LPS is an endotoxin present on the outer cell membrane of Gram negative bacteria, and one of the most used agents as a direct activator of the immune system cells, including microglial cells. Several studies show that LPS is neurotoxic *in vivo* and *in vitro* only in the presence of microglia. Therefore, cultured retinal explants were exposed to 3 μg/ml LPS for 24 hours, and the expression profile of NPY and NPY receptors was evaluated by qRT-PCR before and after LPS exposure. NPY and Y_1R, Y_2R and Y_5R mRNAs were detected in cultured retinal explants (Fig. 14). Next, we assessed whether LPS exposure could alter the mRNA levels of NPY and NPY receptors in retinal explants. Upon exposure to LPS the expression of NPY mRNA decreased to 0.40±0.09 ($p=0.037$, $n=10$) (Fig. 14).

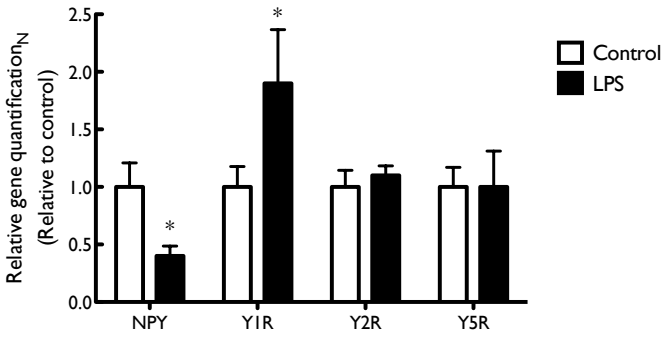

Figure 14. LPS decreases NPY mRNA and increases Y_1R mRNA expression in cultured retinal explants. The mRNA expression of NPY, Y_1R, Y_2R and Y_5R was assessed by qRT-PCR. Retinal explants were cultured for 4 days and exposed to LPS (3 μg/ml) during 24 h. The results are expressed relatively to the control condition (untreated explants), and data are presented as mean ± SEM of 10-12 explants. *p<0.05, significantly different from control condition; statistical significance was analyzed by the unpaired Student's t-test with Welch's correction.

On the opposite, the levels of Y_1R mRNA increased to 1.90±0.47 (p=0.048, n=12), upon exposure of retinal explants to LPS (Fig. 14). No significant differences were detected on the expression of Y_2R (p=0.553, n=10) and Y_5R (p=0.699, n=12) mRNA after exposure to LPS in retinal explants (Fig. 14).

3.3. NPY inhibits the alterations in retinal microglia morphology via Y_1R activation

Activation of microglia is commonly followed by modifications in their morphology from a ramified to a more amoeboid cell shape, being this one of the hallmarks of microglia activation profile (Kettenmann et al., 2011). Having found that NPY and Y_1R expression is altered in retinal explants upon LPS exposure, we next tested whether the morphological transition of microglia triggered by LPS could be inhibited by NPY, and evaluated the involvement of Y_1R in cultured retinal explants. We have performed a morphometric analysis of microglial cells in cultured retinal explants, measuring the area, perimeter, Feret's maximum diameter and circularity of microglial cells. Analysis of microglial cells upon exposure to 3 μg/ml LPS for 24 h indicated a significant decrease in cell perimeter to 89.06±3.98 μm (p=0.040, n=4) (Fig. 15B) and in Feret's maximum diameter to 21.88±0.42 μm (p=0.017, n=4), a measure of cell length (Fig. 15C). Although the area of microglial cells remained unchanged (227.70±13.89 μm^2; p=0.603, n=7) (Fig. 15A) the circularity index significantly increased after LPS exposure to 0.32±0.01 (p<0.001, n=4), consistent with a transformation of ramified to ameboid morphology (Fig. 15D).

To evaluate if NPY could inhibit the morphological changes in microglia triggered by LPS, the retinal explants were exposed to NPY (1 μM) 1 h before incubation with LPS. NPY inhibited the decrease in the perimeter of microglial cells (131.40±7.17 μm; p=0.008, n=4) and the decrease in Feret's maximum diameter (25.89±0.55 μm; p=0.040, n=4), in explants treated with LPS (Figs. 15B and C, respectively). The circularity index of microglial cells in explants treated with NPY was significantly lower (0.16±0.02; p<0.001, n=4), when compared with microglial cells from explants incubated with LPS alone (Fig. 15D), indicating a more ramified microglia morphology. The area of microglial cells remained unchanged (159.20±11.88 μm^2; p=0.366, n=4) (Fig. 15A). The parameters evaluated in retinal explants treated with NPY before incubation with LPS were not significantly different from the control group.

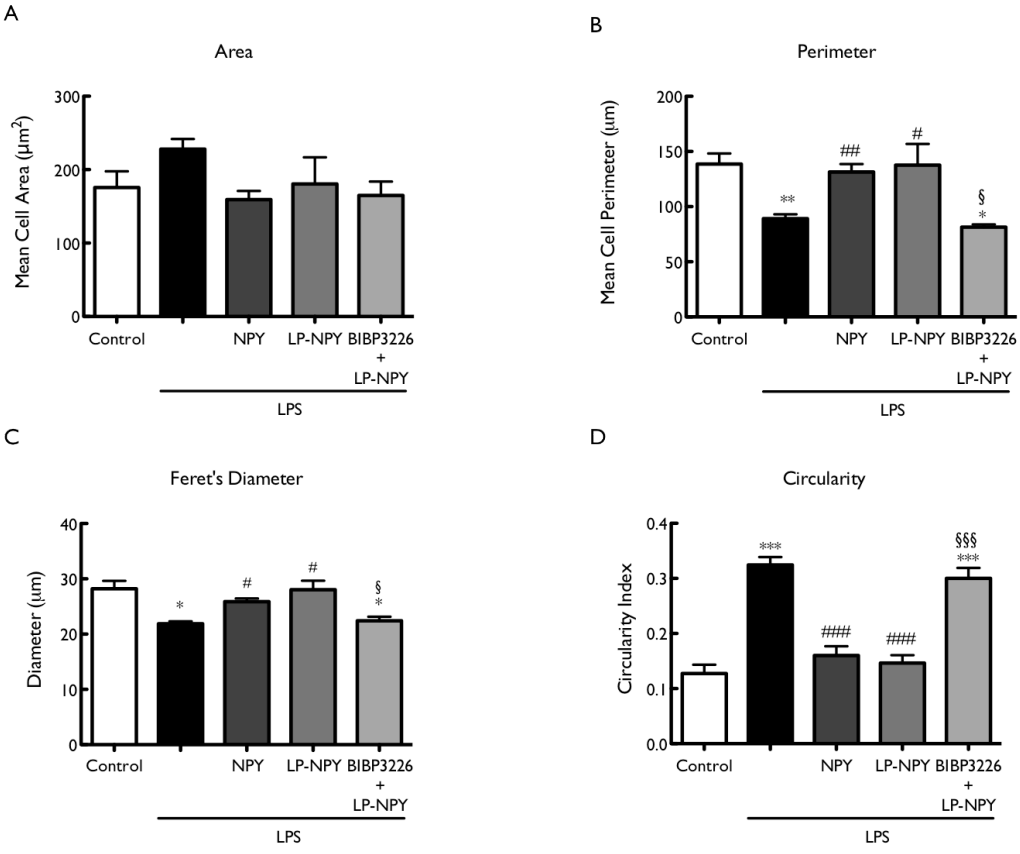

Figure 15. NPY inhibits morphological changes in retinal microglia via Y_1R activation. Morphometric analysis of microglial cells immunoreactive for CD11b in cultured retinal explants. Retinal explants were cultured for 4 days and were exposed to LPS (3 μg/ml) at DIV 3 for 24 h in the absence or presence of 1 μM NPY, 1 μM [Leu^{31}, Pro^{34}]-NPY and/or 1 μM BIBP3226. Data are presented as mean ± SEM. Eight random fields per each explant (2 field per retinal quadrant) were analyzed (40x magnification) containing between 8-15 microglial cells/field (n = 4-7 explants for each condition). *p<0.05, **p<0.01, ***p<0.001, significantly different from control; #p<0.05, ##p<0.01, ###p<0.001, significantly different from LPS condition; §p<0.05, §§§p<0.001, significantly different from LPS + [Leu^{31}, Pro^{34}]-NPY; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

In order to investigate the potential involvement of Y_1R on the inhibition of morphological changes of retinal microglial cells to ameboid shape, retinal explants were

exposed to a Y_1R agonist (LP-NPY, 1 μM), 1 h before exposure to 3 μg/ml LPS. Microglial cells perimeter was significantly higher (137.70±19.17 μm; p=0,046, n=4), when compared to the LPS condition (Fig. 15B). LP-NPY inhibited the decrease in Feret's maximum diameter (28.04±1.64 μm; p=0.014, n=5) (Fig. 15C) and the increase in microglia circularity index (0.15±0.01; p<0.001, n=4) (Fig. 15D), induced by LPS. No significant changes were found in microglia area (180.40±36.37 μm^2; p>0.999, n=4), when comparing with microglia from explants exposed to LPS (Fig. 15A). In addition, to further confirm that the NPY induced inhibition of morphological changes in retinal microglia was mediated via Y_1R, a selective antagonist for Y_1R (BIBP3226) was used. Explants were pre-treated with 1 μM BIBP3226 30 min before treatment with 1 μM LP-NPY. In fact, when Y_1R was blocked, microglia in retinal explants exposed to 3 μg/ml LPS showed a significant lower cell perimeter (81.40±2.41 μm) when comparing with the control group (p=0.014, n=4), and when comparing with explants treated with LPS plus LP-NPY (p=0.016, n=4) (Fig. 15B). Furthermore, microglial cells Feret's maximum diameter was significantly decreased when comparing with the control group (22.43±0.73 μm; p=0.034, n=4) and when comparing with explants treated with LPS plus LP-NPY (p=0.029, n=4) (Fig. 15C). The circularity index was significantly increased when comparing with the control group (0.30±0.02; p<0.001, n=4) and with explants treated with LPS plus LP-NPY (p<0.001, n=4) (Fig. 15D). There were no significant changes in the area of microglial cells in explants pre-treated with BIBP3226 (164.80±18.83 μm^2; p>0.999, n=4), when comparing with microglia from untreated explants and from explants exposed to LPS and LP-NPY (Fig. 15A).

3.4. NPY inhibits iNOS expression in retinal microglial cells

Inducible nitric oxide synthase (iNOS) or type II NOS is an enzyme responsible for the production of NO, with conversion of L-arginine to L-citrulline in the presence of oxygen and cofactors. The overproduction of NO by iNOS has been claimed to be involved in apoptosis following inflammation and ischemia in the retina (Cho et al., 2011). The expression of iNOS is induced in microglial cells by exposure to LPS and cytokines. In order to determine whether NPY could inhibit NO production after LPS exposure, we evaluated the expression of iNOS in microglial cells in cultured retinal explants. Following LPS exposure, it was found that iNOS-IR significantly increased to 20,19±2,13 (p<0.001, n=5) in microglial cells as compared to control (Figs. 16A and B). Treatment of retinal explants with 1 μM NPY 1 h before incubation with LPS significantly decreased iNOS-IR (12,36±2,20;

p=0.025, n=6) in microglial cells (Figs. 16A and B), when compared with microglial cells from explants exposed to LPS.

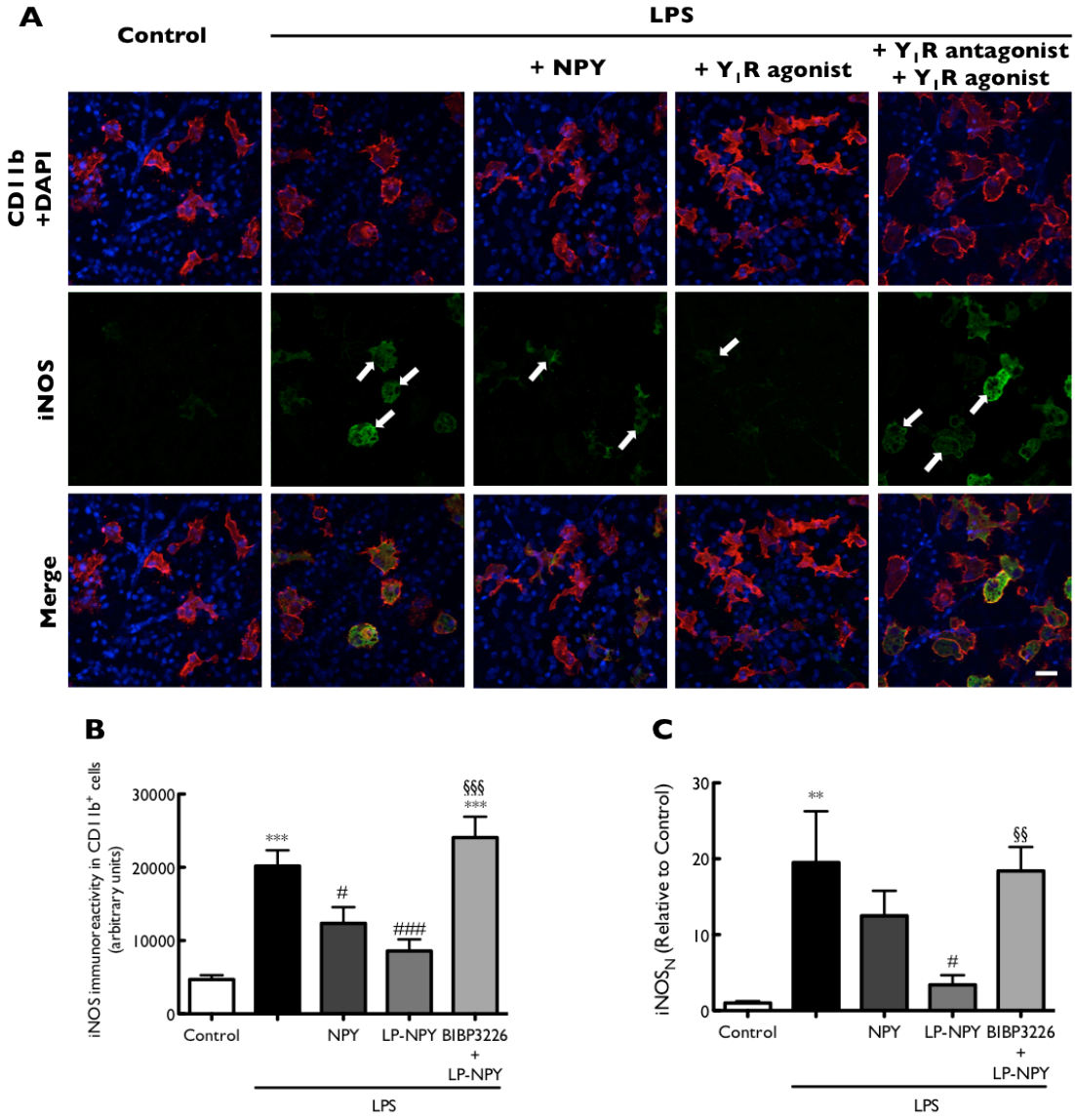

Figure 16. NPY inhibits iNOS immunoreactivity via Y_1R activation in retinal microglial cells in cultured retinal explants. The iNOS expression was evaluated in microglia in cultured retinal explants. Retinal explants were cultured for 4 days and were exposed to LPS (3 μg/ml) at DIV 3 for 24 h in the absence or presence of 1 μM NPY, 1 μM [Leu^{31}, Pro^{34}]-NPY or 1 μM BIBP3226. (A) Immunohistochemistry was performed using antibodies against CD11b (red) and iNOS (green; arrows). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (B) The iNOS immunoreactivity was quantified in microglia in retinal explants. Data are presented as mean ± SEM of 5-6 explants in arbitrary fluorescence units and represent the iNOS immunoreactivity in microglial cells (CD11b+) quantified on 12 random fields (40x magnification) comprising four retinal quadrants (3 fields per retinal quadrant). (C) The mRNA expression of iNOS was assessed by RT-qPCR in cultured retinal explants. The results are expressed relatively to the control condition (untreated explants), and data are presented as mean ± SEM of 4-11 explants. **p<0.01, ***p<0.001, significantly different from control; #p<0.05, ###p<0.001, significantly different from LPS condition; §p<0.05, §§p<0.01, significantly different from LPS + [Leu^{31},Pro^{34}]-NPY; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

Additionally, we tested the possible involvement of Y_1R on the inhibition of iNOS expression in retinal microglia. Thus, retinal explants were treated with 1 μM LP-NPY 1 h before exposure to LPS and iNOS-IR significantly decreased to 8,58±1,57 in retinal

microglial cells ($p<0.001$, n=6), as compared with microglia from explants exposed to LPS (Figs. 16A and B). The effect of LP-NPY on iNOS expression was abolished when retinal explants were pre-treated with the Y_1R antagonist, BIBP3226 (24,08±2,84; $p<0.001$, n=6) (Figs. 16A and B), thus confirming the role of Y_1R on the inhibition of iNOS expression in retinal microglia. These results were further confirmed by qRT-PCR. Upon exposure to LPS, iNOS mRNA expression on retinal explants significantly increased to 19.50±6.76 when comparing with the control group ($p=0.003$, n=7) (Fig. 16C). However, by qRT-PCR no significant changes were found in iNOS mRNA expression in explants exposed to LPS in the presence of NPY (12.50±3.28; $p>0.999$, n=7), when comparing to explants exposed to LPS alone (Fig. 16C). Furthermore, iNOS mRNA expression decreased to 3.40±1.26 in retinal explants treated with LP-NPY ($p=0.028$, n=7), when compared with the LPS condition (Fig. 16C). When retinal explants were pre-treated with the Y_1R antagonist, BIBP3226, the effect of LP-NPY on iNOS mRNA expression was blocked (18.40±3.16; $p=0.001$, n=4) (Fig. 16C).

We further confirmed these results in primary cultures of retinal neural cells. After incubation with 1 μg/ml LPS for 24 h, iNOS-IR increased in microglial cells (Fig. 17; arrows) as compared with the control group. In cultures treated with 1 μM NPY or 1 μM LP-NPY 1 h before exposure to LPS, iNOS-IR in retinal microglia did not increase and was similar to control (Fig. 17). The protective effect of LP-NPY was blocked by pre-treating the retinal cultures with a selective Y_1R antagonist (1 μM BIBP3226). Under these conditions, iNOS-IR in retinal microglia was similar to LPS condition (Fig. 17; arrows).

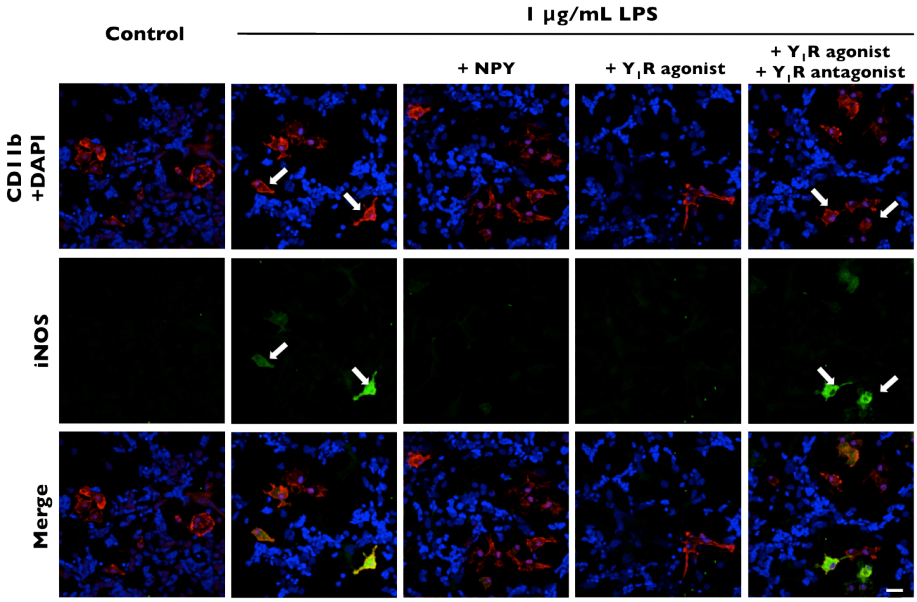

Figure 17. NPY inhibits iNOS immunoreactivity via Y_1R activation in retinal microglial cells in primary retinal cell cultures. The iNOS expression was evaluated in microglia in primary cultures of retinal neural cells. Cells were cultured for 7 days and exposed to LPS (1 μg/ml) at DIV 6 for 24 h in the absence or presence of 1 μM NPY, 1 μM [Leu^{31}, Pro^{34}]-NPY or 1 μM BIBP3226. Immunohistochemistry was performed using antibodies against CD11b (red) and iNOS (green; arrows). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

3.5. NPY decreases ROS production in retinal microglia exposed to LPS

Several reports describe that exposure of microglia to LPS induces stimulation of NAPH oxidase activity and the subsequent production of ROS by microglial cells, including superoxide anion (O_2^-), hydroxyl free radical and hydrogen peroxide (Block et al., 2007, Langmann, 2007, Ibrahim et al., 2011).

The effect of NPY in the production of O_2^- by microglia was assessed by monitoring dihydroethidium (DHE)-derived fluorescence in retinal explants. This fluorescent probe has the ability to freely permeate cell membranes and to display superoxide production. In control conditions, DHE fluorescence was almost undetectable, indicating that the production of superoxide anion in basal condition is very low (Fig. 18). Upon LPS incubation, the DHE fluorescence increased to 19.91±1.18 ($p<0.001$, n=6), particularly in microglial cells (CD11b-positive cells) (Figs. 18A and B). When cultured retinal explants were treated with 1 μM NPY the LPS-induced increase in DHE fluorescence in microglial cells was inhibited to values similar to control (2.70±0.41; $p<0.001$, n=7) (Fig. 18).

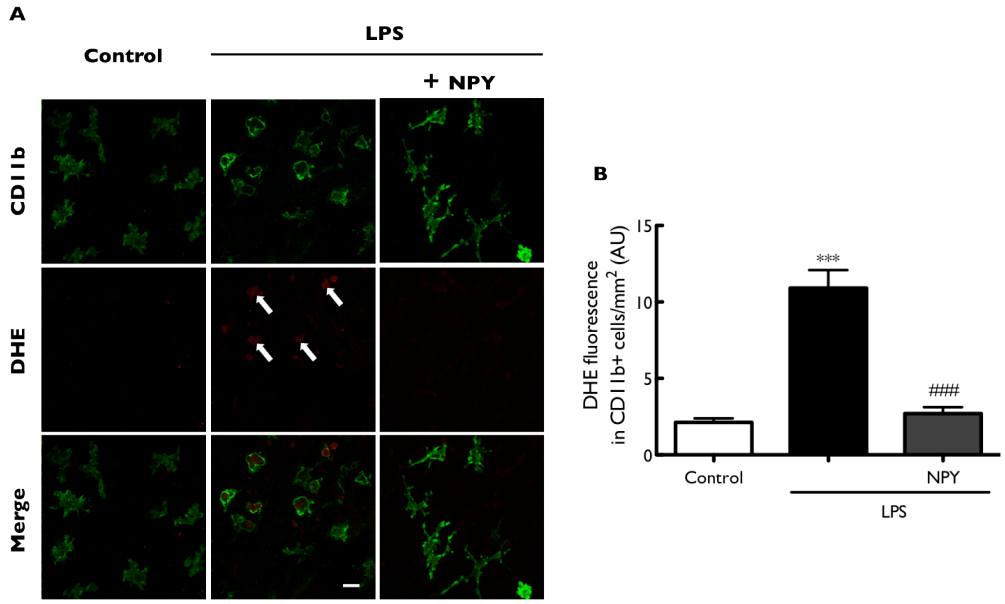

Figure 18. NPY inhibits ROS production in retinal microglia in cultured retinal explants exposed to LPS. The DHE fluorescence was monitored in microglia in cultured retinal explants. Retinal explants were cultured for 4 days and exposed to LPS (3 μg/ml) at DIV 3 for 24 h in the absence or presence of 1 μM NPY. (A) DHE fluorescence (red; arrows) was evaluated in microglia (CD11b+ cells; green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (B) The DHE fluorescence was quantified in microglial cells in retinal explants. The results are expressed in arbitrary fluorescence units (AU), and data are presented as mean ± SEM of 6-7 explants, representing the DHE fluorescence in microglia on twelve random fields (40x magnification) in four retinal quadrants (3 fields per retinal quadrant). ***$p<0.001$, significantly different from control; ###$p<0.001$, significantly different from LPS condition; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

To confirm that superoxide production was derived mainly from microglial cells, we used cultures of purified retinal microglial cells. When cells were incubated with 1 μg/ml LPS, DHE fluorescence increased in the soma of microglial cells (Fig. 19A, arrows), although not significantly. Upon treatment with 1 μM NPY, the increase in DHE fluorescence in microglia triggered by exposure to LPS was inhibited (Figs. 19A and B) indicating a potential role of NPY in the modulation of superoxide production in microglial cells.

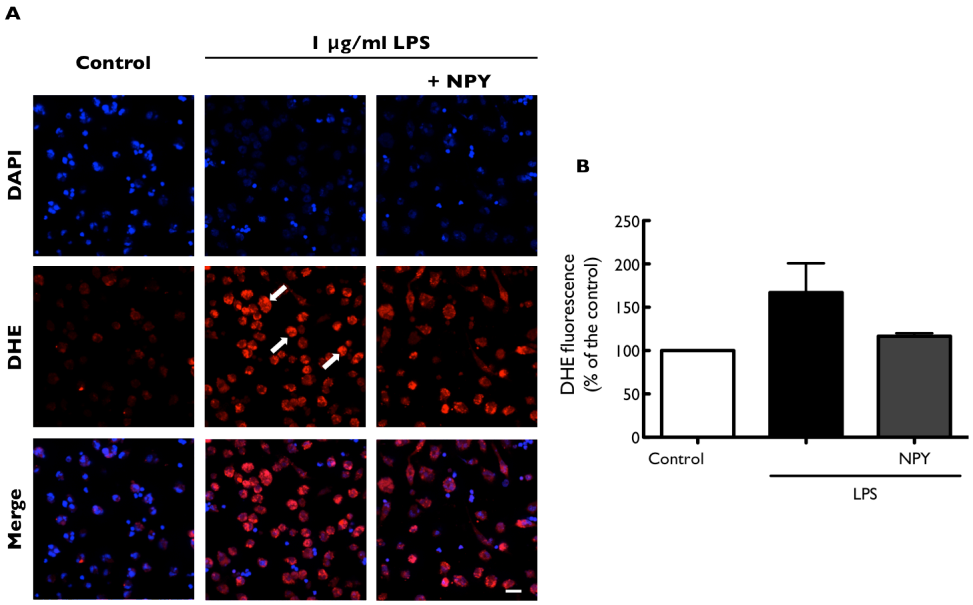

Figure 19. NPY inhibits ROS production in purified retinal microglial cell cultures exposed to LPS. The DHE fluorescence was monitored in microglia in cultures of purified microglial cells. Microglia cultures were cultured for 3 days and exposed to LPS (1 μg/ml) at DIV 2 for 24 h in the absence or presence of 1 μM NPY. (A) DHE fluorescence (red; arrows). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (B) The DHE fluorescence was quantified in microglial cells. The results are expressed in percentage of DHE fluorescence relatively to the control (untretaed cultures), and data are presented as mean ± SEM of 1-3 retinal microglia cultures.

3.6. Y_1 receptor activation decreases TNF-α and IL-1β mRNA expression in cultured retinal explants

Microglia-mediated neurotoxicity can be triggered by LPS and other stimuli, and is characterized by the release of neurotoxic factors, such as pro-inflammatory cytokines (Lull and Block, 2010). The release of TNF-α, IL-1β and IL-6 by microglia can be used as a marker of microglia shift to an activated phenotype in the retina (Schuetz and Thanos, 2004).

Therefore, cultured retinal explants were exposed to 3 μg/ml LPS for 24 h in the absence or presence of 1 μM NPY, 1 μM [Leu^{31}, Pro^{34}]-NPY or 1 μM BIBP3226, and the expression profile of pro-inflammatory cytokines was evaluated by qRT-PCR. Incubation of retinal explants with LPS increased the mRNA expression of TNF-α (9.30±3.46; p=0.026, n=8), IL-1β (8.50±1.21; p<0.001, n=7) and IL-6 (10.40±3.73; p=0.040, n=8) (Figs. 20A, B and C, respectively), when compared with the control condition. Pre-treatment of retinal

explants with NPY decreased the mRNA expression of IL-1β to 4.30±0.60 (p=0.036, n=7) (Fig. 20B), but not TNF-α (3.50±1.05; p=0.151, n=10) (Fig. 20A) and IL-6 (7.70±2.09; p>0.999, n=10) (Fig. 20C). However, although pre-treatment with Y_1R agonist did not decrease IL-6 mRNA expression (4.00±0.65; p=0.513, n=9) (Fig. 20C), Y_1R activation decreased mRNA expression of TNF-α to 2.00±0.42 (p=0.041, n=8) and IL-1β to 4.40±1.59 (p=0.049, n=7) (Figs. 20A and B, respectively). Incubation of retinal explants with Y_1R selective antagonist (BIBP3226; 1 μM) blocked the decrease in mRNA expression of IL-1β (12.30±0.63; p=0.035, n=4) (Fig. 20B), and had no effect on the expression of TNF-α (4.20±3.98; p=0.228, n=4) and IL-6 mRNA (5.80±1.67; p=0.264, n=4) (Figs. 20A and C, respectively).

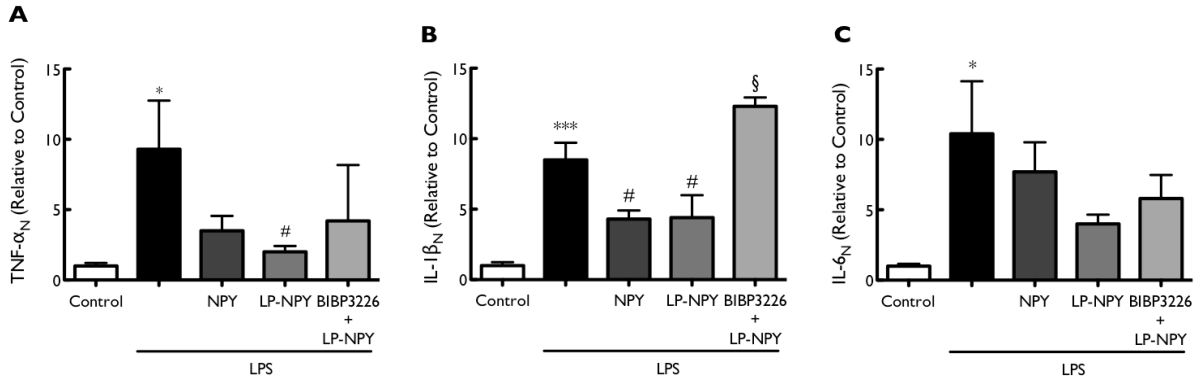

Figure 20. Y_1 receptor activation inhibits the increase in TNF-α and IL-1β mRNA expression triggered by LPS in cultured retinal explants. The mRNA expression of TNF-α (A), IL1-β (B), and IL-6 (C) was assessed by RT-qPCR in cultured retinal explants. Explants were cultured for 4 days and were exposed to LPS (3 μg/ml) at DIV 3 for 24 h. NPY (1 μM) or [Leu^{31}, Pro^{34}]-NPY (1 μM) were incubated 1 h before exposure to LPS. When present, BIBP3226 (1 μM) was added 30 min before the treatment with [Leu^{31}, Pro^{34}]-NPY. The results are expressed relatively to the control condition (untreated cultures), and data are presented as mean ± SEM of 4-11 explants. *p<0.05, ***p<0.001, significantly different from control; #p<0.05, significantly different from LPS; §p<0.05, significantly different from LPS + [Leu^{31},Pro^{34}]-NPY; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.7. Y_1 receptor activation decreases pro-inflammatory cytokine release in cultured retinal explants

After assessing the gene expression level, we investigated the levels of pro-inflammatory cytokines released in retinal explants upon LPS exposure. For this purpose, the levels of TNF-α, IL-1β and IL-6 were measured by ELISA in the culture medium of retinal explants after 4 days in culture. Exposure of retinal explants to 3 μg/ml LPS for 24 h increased the levels of TNF-α (192.80±25.02 pg/ml; p<0.001, n=14), IL-1β (213.60±13.88 pg/ml; p<0.001, n=13) and IL-6 (845.90±98.58 pg/ml; p<0.001, n=13), when compared with the control group (Figs. 21A, B and C, respectively). When the explants were treated with 1

μM NPY the levels of IL-1β and IL-6 in the medium decreased to 123.50±8.88 pg/ml (p=0.025, n=13) and 390.60±92.86 pg/ml (p=0.003, n=8), respectively, comparing with the LPS condition (Figs. 21B and C, respectively). NPY was unable to decrease the release of TNF-α (216.10±26.13 pg/ml; p>0.999, n=17) upon LPS exposure (Fig. 21A). Pre-treatment of explants with 1 μM [Leu^{31}, Pro^{34}]-NPY decreased the levels of TNF-α (92.07±9.11 pg/ml p=0.005, n=15), IL-1β (91.05±7.50 pg/ml; p=0.012, n=6) and IL-6 (263.60±62.34 pg/ml; p<0.001, n=8), when compared to explants incubated with LPS alone (Figs. 21A, B and C, respectively). The inhibitory effect of LP-NPY on the release of pro-inflammatory cytokines was blocked when retinal explants were pre-treated with the Y_1R antagonist, BIBP3226 (1 μM) (Figs. 21A, B and C). These results suggest that the activation of Y_1R decreases pro-inflammatory cytokine production triggered by LPS.

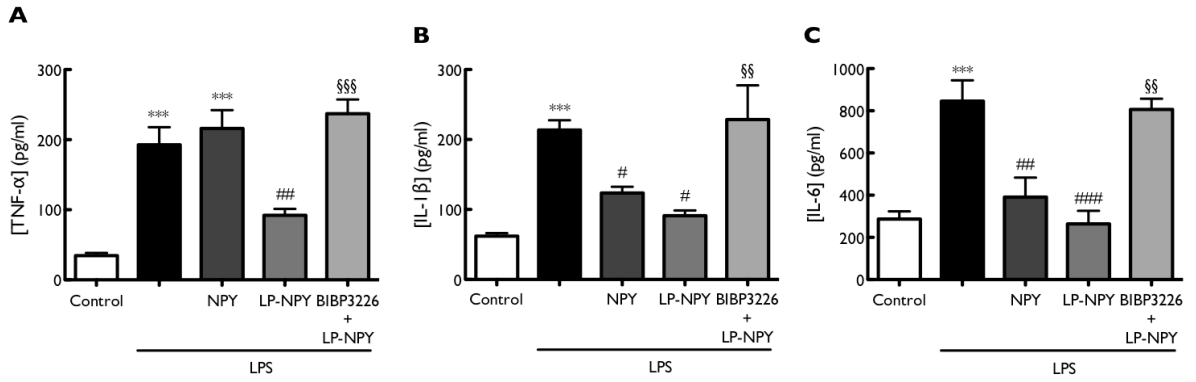

Figure 21. Y_1 receptor activation inhibits the increase in pro-inflammatory cytokine levels triggered by LPS in cultured retinal explants. The production of TNF-α (A), IL-1β (B), and IL-6 (C) was measured by ELISA in cultured retinal explants. Explants were exposed to LPS (3 μg/ml) at DIV 3 for 24 h. NPY (1 μM) or [Leu^{31}, Pro^{34}]-NPY (1 μM) were incubated 1 h before exposure to LPS. When present, BIBP3226 (1 μM) was added 30 min before the treatment with [Leu^{31}, Pro^{34}]-NPY. The results are expressed in picogram of protein (for each cytokine) per milliliter of culture medium, and data are presented as mean ± SEM of 6-17 explants. ***p<0.001, significantly different from control; #p<0.05, ##p<0.01, ###p<0.001, significantly different from LPS; §§p<0.01, §§§p<0.001, significantly different from LPS + [Leu^{31},Pro^{34}]-NPY; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.8. Y_1R activation prevents the increase in the number of ED-1-, but not OX-6-positive microglial cells in the retina after I/R

Based on the previous results, we decided to unveil the potential protective effect of NPY using an animal model of retinal degeneration. Thus, we first started assessing microglia activation in a model of retinal ischemia-reperfusion (I/R) injury. For that, one eye of the rat was subjected to ischemia during 60 min. The contralateral eye served as control. Both eyes were intravitreally injected with 0.9% NaCl (control group), 10 μg NPY or 10 μg [Leu^{31}, Pro^{34}]-NPY 2 h before retinal ischemia. After ischemia, a 24 h period of reperfusion was

allowed. First, we evaluated ED-1 and OX-6 immunoreactivity in microglial cells (Iba-1$^+$ cells) in retinal slices. Retinal I/R injury induced an 11.8 and 11.1-fold increase in the number of ED-1 (85.32±3.20%; p<0.001, n=5) and OX-6-positive (48.32±7.15%; p<0.001, n=5) microglial cells, respectively, in the ischemic retinas compared to non-ischemic retinas (Figs. 22A and B, respectively). ED-1 and OX-6-positive cells were present in very low amount in non-ischemic retinas (Figs. 22A and B, respectively), confirming that these markers are mainly expressed by activated microglia. The intravitreal injection of NPY before I/R injury did not significantly change the percentage of ED-1 (75.32±5.25%; p=0.157, n=6) and OX-6-positive (33.88±6.23%; p=0.160, n=6) microglial cells, when compared to the saline-treated group (Figs. 22A and B, respectively). Interestingly, intravitreal injection of Y_1R agonist before I/R significantly inhibited the increase in the percentage of ED-1-positive cells induced by I/R (60.70±6.79; p=0.011, n=5) (Fig. 22A), but not the percentage of OX-6-positive microglial cells (38.56±11.32%; p=0.487, n=5) (Fig. 22B).

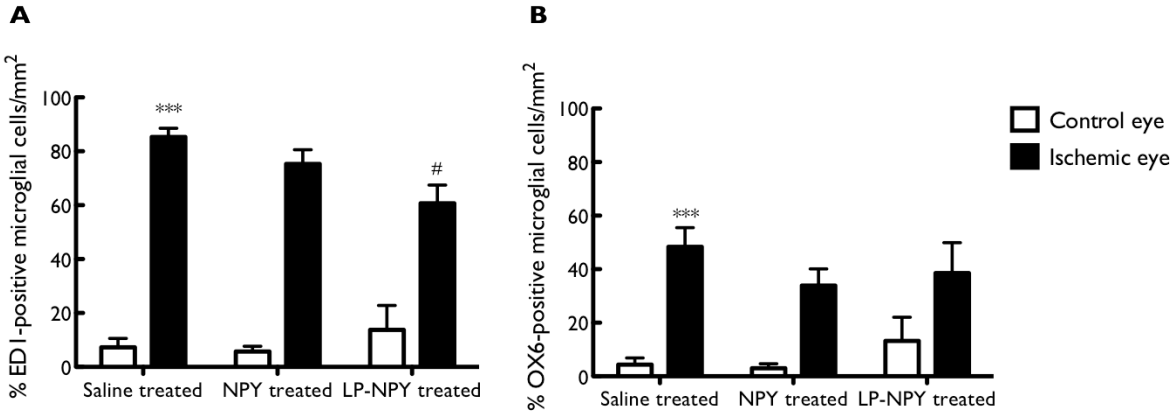

Figure 22. Y_1R activation inhibits the increase in the number of ED-1-positive microglial cells triggered by I/R. The number of microglial cells (Iba-1-positive) immunoreactive for ED-1 (A) and OX-6 (B) was quantified in frozen retinal sections. Both eyes were intravitreally injected with 0.9 % NaCl (control group), 10 μg NPY or 10 μg [Leu^{31}, Pro^{34}]-NPY, 2 h before retinal ischemia. After 60 min of ischemia there was a 24-h period of reperfusion. The results are expressed as the percentage of ED-1- and OX-6-positive microglial cells/mm^2, and data are presented as mean ± SEM of 5-6 independent experiments, representing eight non-consecutive transverse retinal sections per eye and ten random fields (40x magnification) per retinal section. ***p<0.001, significantly different from the non-ischemic eye; #p<0.05, significantly different from the ischemic retinas in the control group; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.9. NPY inhibits microglia activation induced by I/R injury through Y_1R activation

In response to neuronal injury, microglia may undergo morphological changes from the so-called resting state, with ramified processes, into an ameboid shape, with short or non-existent processes. Thus, we evaluated the changes in microglia (Iba-1$^+$ cells) morphology

after I/R injury. The percentage of ameboid microglial cells in the ischemic retinas was 16.2-fold higher compared with non-ischemic retinas (95.38±1.98%; p<0.001, n=5) (Fig. 23). Treatment with NPY before I/R injury inhibited the increase in the percentage of ameboid microglial cells in the ischemic retinas (69.03±8.40%; p=0.021, n=6), compared with the saline-treated group (Fig. 23). Moreover, intravitreal injection of Y_1R agonist before I/R injury also inhibited the increase in the percentage of ameboid microglial cells (55.30±12.60%; p=0.014, n=5), when compared to the control group (Fig. 23), thus confirming that activation of Y_1R can modulate microglia activation after I/R injury.

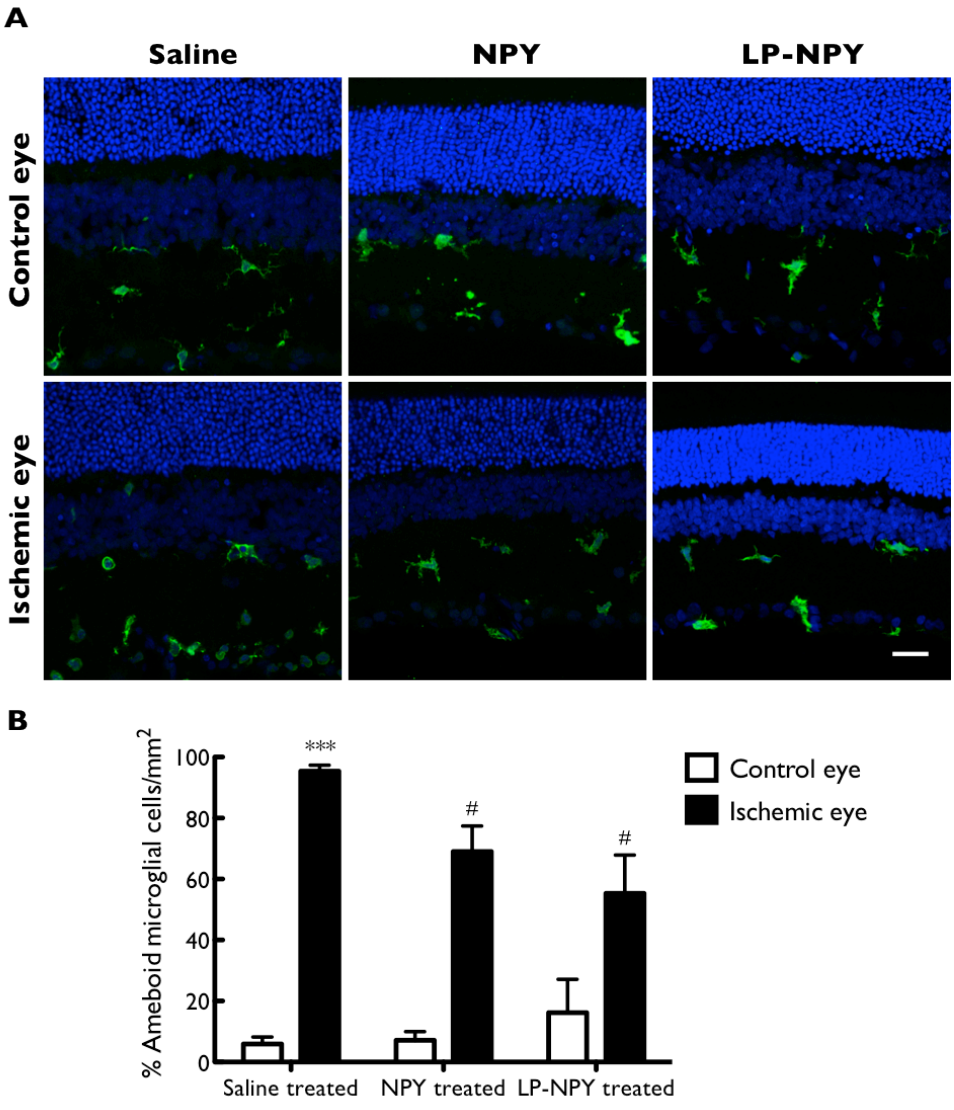

Figure 23. NPY inhibits microglia activation triggered by I/R injury through Y_1R activation. The number of ameboid microglial cells (Iba-1-positive) was quantified in frozen retinal sections. Both eyes were intravitreally injected with 0.9 % NaCl (control group), 10 μg NPY or 10 μg [Leu^{31}, Pro^{34}]-NPY, 2 h before retinal ischemia. After 60 min of ischemia there was a 24-hour period of reperfusion. (A) Microglia morphology was evaluated in Iba-1-positive cells (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (B) The results are expressed as the percentage of ameboid microglial cells/mm^2, and data are presented as mean ± SEM of 5-6 independent experiments, representing eight non-consecutive transverse retinal sections per eye and ten random fields (40x magnification) per retinal section. ***p<0.001, significantly different from the non-ischemic retinas; #p<0.05, significantly different from the ischemic retinas in the control group; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.10. NPY has no inhibitory effects on the production of pro-inflammatory cytokines in ischemic retinas after 24h reperfusion

After assessing microglia activation in a model of retinal I/R injury, we evaluated the production of pro-inflammatory cytokines after I/R. The production of TNF-α, IL-1β and IL-6 was measured by ELISA after 24 h of reperfusion. In fact, retinal I/R injury did not induce a significant increase on the production of pro-inflammatory cytokines after 24 h of reperfusion, compared to non-ischemic retinas (Figs. 24A, B and C). Moreover, the intravitreal injection of NPY (10 μg) did not induce any significant effect on the production of pro-inflammatory cytokines either in the saline treated eyes or in the ischemic eyes (Figs. 24A, B and C).

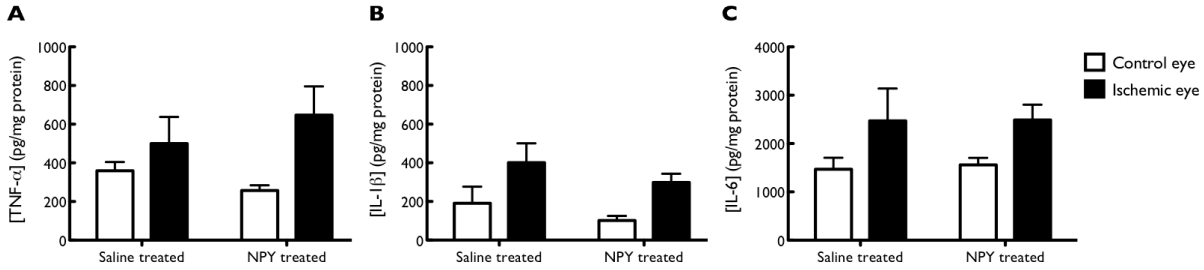

Figure 24. NPY does not significantly affect the production of pro-inflammatory cytokines in ischemic retinas after 24h reperfusion. The production of TNF-α (A), IL-1β (B), and IL-6 (C) was monitored by ELISA in retinas following I/R injury. Both eyes were intravitreally injected with 0.9% NaCl (control group) or 10 μg NPY, 2 h before retinal ischemia. After 60 min of ischemia there was a 24-hour period of reperfusion. The results are expressed in picogram of cytokine per milliliter and per milligram of protein, and data are presented as mean ± SEM of 4-6 retinas. Statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.11. I/R injury increases NPY and Y_2R mRNA expression in the rat retina

The mRNA expression of NPY and NPY receptors was evaluated after I/R injury by qRT-PCR. Because changes in mRNA expression normally occur early in time after an insult, we have also included a group of animals on which an 8-h reperfusion time was applied. After 24 h of reperfusion, the mRNA expression of NPY and NPY receptors was not significantly different between ischemic and non-ischemic retinas (Figs. 25A, B, C and D). Interestingly, the mRNA expression of NPY in ischemic retinas after 8 h reperfusion increased to 1.36±0.12 (p=0.021, n=6), when compared with non-ischemic retinas (Fig. 25A). Moreover, the mRNA expression of Y_2R was 11-fold higher in ischemic retinas compared

with non-ischemic retinas (p<0.001, n=6) (Fig. 25C). The mRNA levels of Y_1R and Y_5R were not significantly different between ischemic and non-ischemic retinas (Figs. 25B and D, respectively), although there was a trend to a decreased mRNA expression of Y_1R in ischemic retinas compared with non-ischemic retinas (0.67±0.16; p=0.139, n=6) (Fig. 25B).

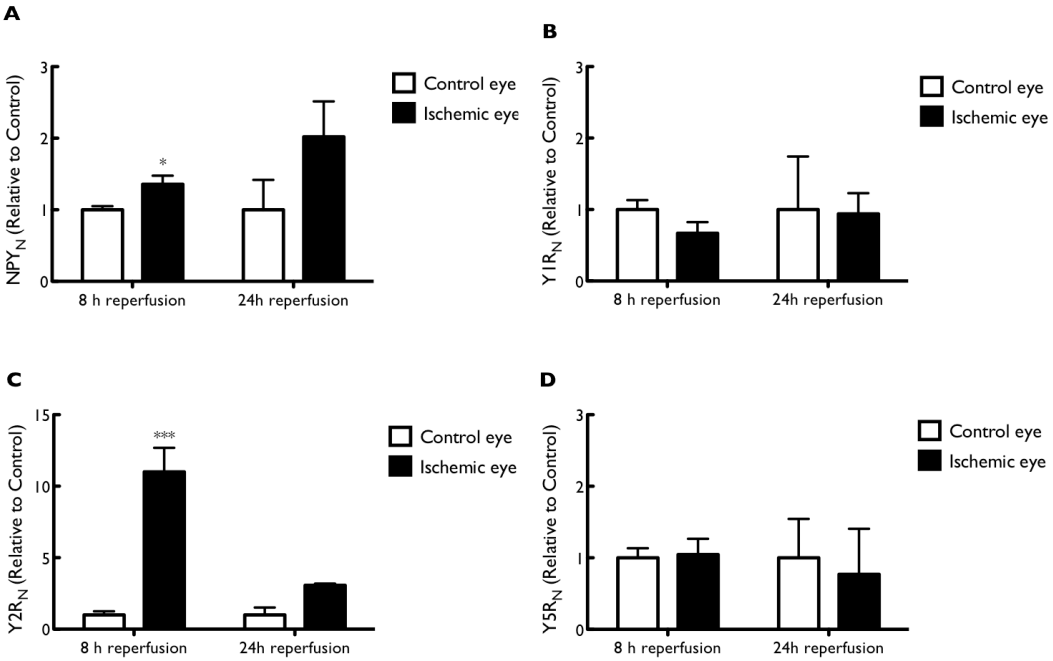

Figure 25. I/R injury induces an increase in mRNA expression of NPY and Y_2R in the rat retina. The mRNA expression of NPY (A), Y_1R (B), Y_2R (C) and Y_5R (D) was assessed by qRT-PCR in retinas. After 60 min of ischemia there was an 8- or 24-h period of reperfusion. The results are expressed relatively to the non-ischemic retinas, and data are presented as mean ± SEM of 6 retinas. *p<0.05, ***p<0.001, significantly different from non-ischemic retinas; statistical significance was analyzed by the unpaired Student's t-test with Welch's correction.

3.12. NPY decreases pro-inflammatory cytokine mRNA expression in ischemic retinas after 8 h reperfusion

The mRNA expression of TNF-α, IL-1β and IL-6 was assessed by qRT-PCR after 8 h of reperfusion. Retinal I/R injury induced a 3.9-, 2.6- and 4.6-fold increase in the mRNA levels of TNF-α (p=0.002, n=6), IL-1β (p=0.004, n=6) and IL-6 (p=0.002, n=6), respectively, when compared with the non-ischemic retinas (Figs. 26A, B and C, respectively). Intravitreal injection of 10 μg NPY significantly decreased the mRNA expression of TNF-α (1.73±0.32; p=0.026, n=6), IL-1β (1.94±0.40; p=0.041, n=6) and IL-6 (1.66±0.37; p=0.002, n=6), when compared with non-ischemic retinas injected with 0.9% NaCl (Figs. 26A, B and C, respectively). Interestingly, pre-treatment of non-ischemic retinas with NPY also decreased IL-1β mRNA expression (0.34±0.09; p=0.041, n=6) compared with the control group (Fig. 26B).

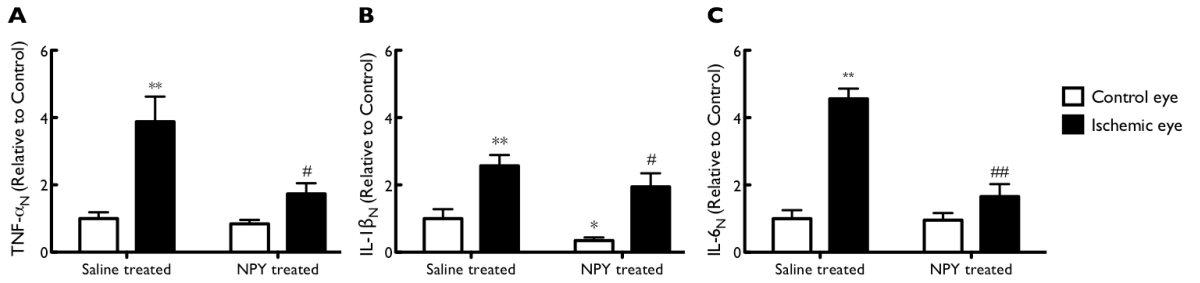

Figure 26. NPY inhibits the increase in pro-inflammatory cytokine mRNA expression in ischemic retinas after 8 h reperfusion. The mRNA expression of TNF-α (A), IL-1β (B), and IL-6 (C) was analyzed by qRT-PCR in retinas. Both eyes were intravitreally injected with 0.9% NaCl (control group) or 10 μg NPY 2 hours before retinal ischemia. After 60 min of ischemia there was an 8-hour period of reperfusion. The results are expressed relatively to the non-ischemic retinas in the control group, and data are presented as mean ± SEM of 6 retinas. *p<0.05, **p<0.01, significantly different from the non-ischemic retinas in the control group; #p<0.05, ##p<0.01, significantly different from the ischemic retinas in the control group; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.13. NPY inhibits the increase in CD11b expression in ischemic retinas after 8h reperfusion

Recent studies describe an association between microglia activation with the increase in the expression level of CD11b (Kettenmann et al., 2011). Thus, the mRNA expression of CD11b was determined by qRT-PCR after 8 h reperfusion. Retinal I/R injury induced a 3.4-fold increase in CD11b mRNA expression compared to non-ischemic retinas (p<0.001, n=6) (Fig. 27). Intravitreal injection of 10 μg NPY partially inhibited the I/R-induced increase in CD11b mRNA expression (2.12±0.26; p=0.0236, n=6) (Fig. 27).

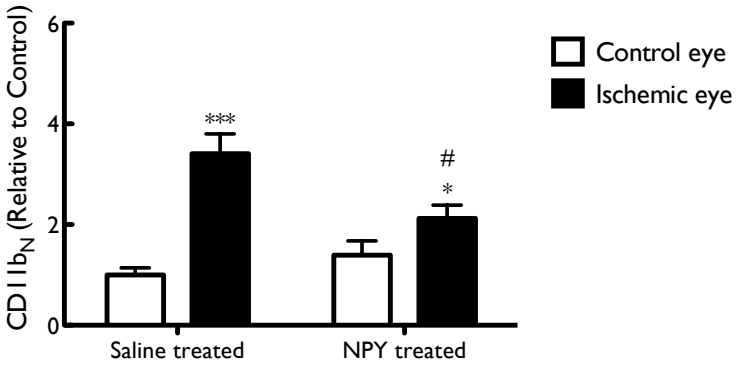

Figure 27. NPY inhibits the increase in CD11b expression in ischemic retinas after 8h reperfusion. The mRNA expression of CD11b was analyzed by qRT-PCR in retinas. Both eyes were intravitreally injected with 0.9% NaCl (control group) or 10 μg NPY, 2 h before retinal ischemia. After 60 min of ischemia there was an 8-hour period of reperfusion. The results are expressed relatively to the non-ischemic retinas in the control group, and data are presented as mean ± SEM of 6 retinas. *p<0.05, ***p<0.001, significantly different from the non-ischemic retinas in the control group; #p<0.05, significantly different from the ischemic retinas in the control group; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

3.14. NPY decreases the production of pro-inflammatory cytokines in ischemic retinas after 8 h reperfusion

After determining the effect of NPY on the mRNA expression of pro-inflammatory cytokines, the effect on cytokines (TNF-α, IL-1β and IL-6) production was also determined by ELISA, after 8 h of reperfusion. The levels of TNF-α (516.30±45.29 pg/mg protein; p<0.001, n=9), IL-1β (1,367.00±182.30 pg/mg protein; p<0.001, n=9) and IL-6 (8,310.00±1,437.00 pg/mg protein; p=0.047, n=9) significantly increased, compared with non-ischemic retinas (Figs. 28A, B and C, respectively). Pre-treatment with 10 μg NPY significantly inhibited the I/R-induced increase in TNF-α (186.90±11.48 pg/mg protein; p<0.001, n=6) and IL-6 (3,742.00±1,065.00 pg/mg protein; p=0.034, n=6) levels in the retina (Figs. 28A and C, respectively). There was a trend for the inhibition of IL-1β levels in ischemic retinas treated with NPY (Fig. 28B), but did not reach statistical significance (1,032.00±57.47 pg/mg protein; p=0.333, n=6).

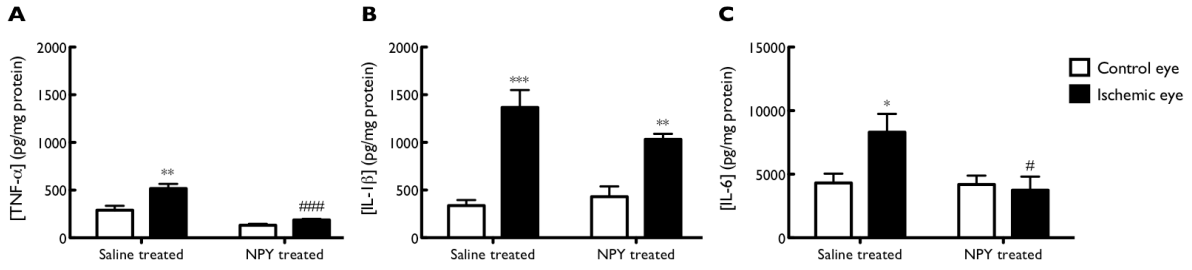

Figure 28. NPY inhibits the increase of pro-inflammatory cytokine levels in ischemic retinas after 8h reperfusion. The levels of TNF-α (A), IL-1β (B), and IL-6 (C) were monitored by ELISA in retinas. Both eyes were intravitreally injected with 0.9% NaCl (control group) or 10 μg NPY, 2 h before retinal ischemia. After 60 min of ischemia there was an 8-hour period of reperfusion. The results are expressed in picogram of cytokine per milliliter and per milligram of protein, and data are presented as mean ± SEM of 6-9 retinas. *p<0.05, **p<0.01, ***p<0.001, significantly different from the non-ischemic retinas in the control group; #p<0.05, ###p<0.001, significantly different from the ischemic retinas in the control group; statistical significance was analyzed by the One-way ANOVA test followed by Bonferroni's multiple comparison post hoc test.

DISCUSSION

4. Discussion

The central nervous system (CNS) is an immune privileged site, due to the presence of the blood brain barrier and blood retinal barrier that limit the access of peripheral immune cells to the brain and retina, respectively, keeping a restricted and controlled microenvironment. However, in response to several insults, such as trauma, infection, toxins and other stimuli, the CNS is capable of activating the innate immune system within the CNS (Lehnardt, 2010, Kraft and Harry, 2011). Indeed, the inflammatory response in the CNS – neuroinflammation - is a process that has been associated with the pathogenesis of several neurodegenerative diseases in the CNS, such as Alzheimer's disease (Hensley, 2010), as well as in retinal degenerative diseases, such as age-related macular degeneration (AMD) (Buschini et al., 2011), diabetic retinopathy (Kumar et al., 2013) and glaucoma (Jiang et al., 2010).

Neuroinflammation is a homeostatic response to brain and retina injury that triggers the activation of several cellular immune mediators (Streit et al., 2004, O'Callaghan et al., 2008). Thus, the neuroinflammatory process includes activation of the immunocompetent cells of the CNS, microglia, resulting in the production of inflammatory mediators such as cytokines and chemokines (Block et al., 2007), and activation of their surface receptors (Streit et al., 2004). Moreover, the activation of microglial cells might also lead to oxidative and nitrosative stress which play a major role in neuroinflammatory processes (Block et al., 2007, Brown and Neher, 2010). The activation of microglial cells can have a beneficial or deleterious outcome in CNS, depending on the duration of the inflammatory response and the factors present on the cellular milieu (Walter and Neumann, 2009). Thus, in an early stage and for a short period of time, microglial cells can produce anti-inflammatory cytokines, including IL-10 and TGF-β, neurotrophic factors and phagocyte cell debris (Graeber et al., 2011), and can be neuroprotective, in order to minimize injury and repair the damaged tissue. However, when microglia activation persists after the initial insult or injury, a chronic neuroinflammatory process occurs, which can be associated with neuronal death (Streit et al., 2004, Block et al., 2007). Moreover, hyperactivation of microglia results in the subsequent release of pro-inflammatory mediators perpetuating the inflammatory process, further promoting microglia activation (Block and Hong, 2005).

Retinal degenerative diseases affect millions of people worldwide, causing varying degrees of irreversible vision loss. The pathogenesis of the different retinal degenerative diseases implies a complex interplay of different factors, being characterized in general by the loss of retinal neurons (Schuetz and Thanos, 2004). Recently, it has been reported that the

pathogenesis of several retinal degenerative diseases has a major contribution of inflammation (Gupta et al., 2003, Schuetz and Thanos, 2004, Jiang et al., 2010). It has been reported a contribution of microglia for the pathogenesis of human retinal degenerative diseases, such as retinitis pigmentosa, AMD (Gupta et al., 2003), diabetic retinopathy (Zeng et al., 2008) and glaucoma (Yuan and Neufeld, 2001). For example, in AMD an excessive activation of resident immune cells and accumulation of byproducts triggers chronic inflammation (Buschini et al., 2011). In fact, many recent studies confirm the role of activated microglia as major mediators of retinal neuroinflammation through the release of neurotoxic factors that ultimately lead to degenerative events in the retina. Therefore, modulation of microglia activation in the retina is an attractive therapeutic target for retinal degenerative diseases.

Glaucoma is characterized by retinal ganglion cell (RGC) death, degeneration of optic nerve axons and excavation of the optic nerve head (ONH). RGC loss ultimately leads to irreversible blindness (Agarwal et al., 2009). The upregulation of genes associated with inflammation and antigen presentation was found in glaucomatous eyes (Jiang et al., 2010), confirming the contribution of the neuroinflammatory response in the pathogenesis of glaucoma. Activated microglia have been involved in the pathophysiology of glaucoma, redistributing and accumulating in the central retina and ONH (Yuan and Neufeld, 2001, Bosco et al., 2011). Microglia activation has also been shown to be associated with the degeneration of RGCs in experimental models of glaucoma involving sclerosis of the episcleral veins (Taylor et al., 2011) and in retinal ischemia (Vidal-Sanz et al., 2001). In this way, the control of microglia reactivity might help mitigate RGC loss in glaucoma.

Microglial cells express several neurotransmitters and neuromodulators that are involved in the control of its functions (Pocock and Kettenmann, 2007). NPY is one of these neuromodulators that exerts its effects in the CNS mainly through the activation of four G protein-coupled receptors (Y_1R, Y_2R, Y_4R and Y_5R). This neuropeptide has been associated with neuroprotective effects in CNS, including a protective effect against glutamate-mediated excitotoxicity (Silva et al., 2003b) and against methamphetamine-induced toxicity in the brain (Silva et al., 2003b, Baptista et al., 2012). In addition, NPY has also neuroprotective effects in the retina (Santos-Carvalho et al., 2013b), protecting retinal neurons against an excitotoxic insult.

Growing evidence reveals an important role of NPY in the immune system (Dimitrijevic and Stanojevic, 2013). The modulation of peripheral inflammation by NPY has been demonstrated (Bedoui et al., 2003, Bedoui et al., 2008, Mitic et al., 2011), whereas a few studies have studied the NPYergic system modulatory effects in microglia. In the

periphery, NPY can modulate several phagocytic functions, increasing the adherence (Nave et al., 2004) and chemotaxis (De la Fuente et al., 2001) of macrophages towards the site of inflammation, increasing (De la Fuente et al., 2001) or decreasing (Dimitrijevic et al., 2005) phagocytosis and superoxide radical production, and inhibiting the production of pro-inflammatory cytokines in macrophages (Straub et al., 2000, De la Fuente et al., 2001).

More recently, it has been described a role for NPY in regulating microglia functions. NPY inhibits microglia motility (Ferreira et al., 2012) and phagocytosis (Ferreira et al., 2011), through Y_1R activation. Additionally, activation of Y_1R by NPY inhibits IL-1β release, preventing iNOS expression and NO production in microglial cells (Ferreira et al., 2010). These findings suggest that NPY can potentially control retinal neuroinflammation, acting as a buffer of retinal microglia activation.

To address this hypothesis, first we used an endotoxin-mediated model of retinal neuroinflammation, using LPS in different culture preparations, and second, we used an animal model of retinal degeneration, a retinal ischemia-reperfusion (I/R) injury model, also characterized by an inflammatory response (Dvoriantchikova et al., 2010). Since previous reports describe that modulation of microglia reactivity occurs through activation of Y_1R, the role of NPY and in particular the Y_1R on microglia activation and retinal inflammatory status was evaluated in the different models used. In this study, we have demonstrated that NPY was able to reduce microglia activation and the expression or production of pro-inflammatory cytokines in the inflamed retina.

The presence of NPY and NPY receptors (NPYRs) is well described in several retinal cell types (Straznicky and Hiscock, 1989, Hutsler and Chalupa, 1995, Hokfelt et al., 1998, Sinclair and Nirenberg, 2001), including retinal microglial cells (Alvaro et al., 2007, Santos-Carvalho et al., 2013a). However, to our knowledge, no previous studies have reported a complete characterization of the expression of NPY and NPYRs in retinal microglial cells. Using qRT-PCR and immunohistochemistry analysis, the expression of NPY and NPYRs (Y_1R, Y_2R and Y_5R) in microglial cells was characterized in different culture preparations. The results show that NPY and Y_1R, Y_2R and Y_5R-IR could be found in microglial cells in cultured retinal explants. A similar result was obtained in primary mixed cultures of rat retinal neural cells, in accordance with previous reports (Alvaro et al., 2007, Santos-Carvalho et al., 2013a), and in primary cultures of purified rat retinal microglial cells. Moreover, when cultured retinal explants were challenged with LPS, the mRNA expression of NPY decreased, suggesting that LPS could act as a negative regulator of NPY expression. A study using the N9 microglial cell line has reported that LPS exposure increases NPY cDNA, and treatment with NPY blocks the reported increase (Ferreira et al., 2010). In other study is

described that administration of LPS in rats did not change the levels of mRNAs for NPY in the arcuate nucleus (Sergeyev et al., 2001). The discrepancy observed between ours and these results could be explained, first by the presence of several cell types in cultured retinal explants that might contribute to the total NPY mRNA expression in opposition to a microglia cell line. Secondly, the expression of NPY might be different in the retina and in the brain, and even in different brain regions. Conversely, the mRNA expression of Y_1R increased upon LPS exposure, and this finding is in accordance with a previous report demonstrating that the exposure of a microglial cell line to LPS results in an increase of Y_1R expression, and that NPY treatment inhibits this effect (Ferreira et al., 2010), suggesting that Y_1R modulation may putatively regulate microglia reactivity after exposure to LPS.

In the healthy retina, microglia are continuously surveying the surrounding microenvironment with their long protrusions. Under stress conditions, microglial cells direct their processes towards the injury site before migrating and retracting their processes and adopt an ameboid morphology, producing a large array of cytotoxic and trophic factors (Kettenmann et al., 2011). It has been reported that LPS, by engagement of TLRs, induces signal cascades that will induce morphological changes in microglial cells, expression of new proteins, including iNOS, followed by TNF-α, IL-1β, IL-6 and NO release (Nakamura et al., 1999, Brown and Neher, 2010, Kaur et al., 2013). For example, TNF-α can directly induce RGC death through receptor-mediated caspase activation, mitochondrial dysfunction and oxidative stress (Berger et al., 2008, Tezel, 2008). Alternatively, the release of neurotoxic factors can further activate microglia and astrocytes, amplifying the inflammatory response, indirectly inducing neuronal damage (Block et al., 2007, Langmann, 2007, Berger et al., 2008, Brown and Neher, 2010). Our results show that changes in retinal microglia morphology to an ameboid shape, induced by LPS in cultured retinal explants, were inhibited by NPY. Growing evidence shows the importance of Y_1R in the immune system. Not only Y_1R has been detected in almost every immune cell type examined so far, including lymphocytes, granulocytes, monocytes (Dimitrijevic et al., 2010, Mitic et al., 2011), dendritic cells, macrophages (Bedoui et al., 2003), neutrophils (Bedoui et al., 2008) and microglia (Ferreira et al., 2010, Santos-Carvalho et al., 2013a), but also activation of Y_1R has been described to be involved in the modulation of several microglial functions, such as microglial cell motility (Ferreira et al., 2012), phagocytosis (Ferreira et al., 2011) and the production of pro-inflammatory cytokines (Ferreira et al., 2010). In order to investigate the potential involvement of Y_1R on the inhibition of morphological changes in retinal microglial cells, retinal explants were treated with a Y_1R agonist [Leu^{31}, Pro^{34}]-NPY (LP-NPY) and a selective antagonist for Y_1R, BIBP3226. The inhibitory effect of NPY on microglia morphological

changes to an ameboid shape was mimicked by activation of Y_1R and blocked by Y_1R antagonist, implying that NPY acts mainly through Y_1R to inhibit microglia activation. These results are consistent with a previous report in a N9 microglial cell line showing that NPY inhibited LPS-induced motility of microglial cells via Y_1R (Ferreira et al., 2012). In another study, it was reported an inhibitory effect of NPY on macrophage (Raw 264.7 cell line) chemotaxis induced by *Leishmania major* (Ahmed et al., 1998).

Based on the results obtained in cultured retinal explants, we decided to unveil the potential protective effect of NPY against neuroinflammation using an animal model of retinal degeneration. Thus, microglia activation was also evaluated in a model of retinal I/R injury. It has been described that after I/R activated microglia undertake a morphological transformation from their surveillant ramified state into an ameboid shape and upregulate several surface markers, such as MHC molecules, CD14, and chemokine receptors (Zhang et al., 2005a, Abcouwer et al., 2010). Our results show that I/R upregulated the expression of the lysosomal protein ED-1, which is the rat homolog of human CD68, and the MHC class II molecule, OX-6, in retinal microglial cells. Both markers were mainly expressed by round and ameboid microglial cells in the inner retina, 24 h after ischemic injury. These results are in accordance to what was previously reported by Zhang and collaborators. They reported the peak of microglia morphological transformation to an ameboid shape between 24 to 72 h following ischemia, with a simultaneous increase in the expression of ED-1 and OX-6 surface markers (Zhang et al., 2005a).

Intravitreal injection of NPY before I/R did not affect the increase in the expression of ED-1 and OX-6 in microglia induced by I/R. However, NPY significantly inhibited the increase in the percentage of ameboid microglia after I/R. Interestingly, intravitreal injection of Y_1R agonist [Leu^{31}, Pro^{34}]-NPY before I/R significantly inhibited the increase in the percentage of ED-1-positive cells induced by I/R in the retina. The absence of suppressive effect of NPY on the percentage of ED-1-positive cells could be associated with a high retinal and/or plasma dipeptidyl peptidase 4 (DPP 4) activities in these rats undergoing I/R. It has been reported that some inbred rat strains, show high DPP 4 activity (Karl et al., 2003). Since DPP 4 can regulate NPY proteolytic processing, influencing receptor specificity by cleaving NPY to the N-terminal truncated NPY(3-36) (Mentlein et al., 1993), high DPP 4 activity can induce activation of Y_2R and Y_5R, confining the NPY functions mediated via Y_1R. A previous study suggests that the upregulation of other surface markers, such as integrin CD11b/OX-42 occurs earlier (6 h) after retinal ischemia (Zhang et al., 2005a). The increase in expression of CD11b is considered a marker of microglia activation in the retina (Langmann, 2007) and in the brain (Kettenmann et al., 2011). NPY partially inhibited the

increase in mRNA expression of CD11b in ischemic retinas after 8 h reperfusion, suggesting a decrease in microglia activation.

Expression of iNOS in activated microglia and astrocytes, results in overproduction of NO that induces neuronal apoptosis by causing neuronal mithocondrial dysfunction in the brain (Brown and Neher, 2010). Moreover, NO produced by activated microglia has been described to originate glutamate-induced excitoxicity by causing neuronal depolarization and glutamate release, followed by overactivation of NMDA receptors (Bal-Price and Brown, 2001, Golde et al., 2002). In another study, it has been demonstrated that the increase in iNOS expression in the RGCL and ONH after retinal ischemia-reperfusion may contribute to RGC degeneration and optic nerve damage (Cho et al., 2011). In addition, the expression of iNOS can be induced in microglial cells by exposure to LPS (Nakamura et al., 1999), as well as the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β (Hirsch et al., 2003). Thus, these evidences suggest that inhibition of iNOS expression in activated microglia may be beneficial in protecting retinal neurons from microglial-induced neurotoxicity. To address this hypothesis, we have evaluated iNOS-IR in retinal microglia and iNOS mRNA expression in cultured retinal explants, after LPS exposure, in the presence or absence of NPY. The upregulation of iNOS expression in retinal microglial cells induced by LPS exposure was reduced in the presence of NPY, in cultured retinal explants. In accordance to this observation, NPY also decreases iNOS-IR and protein levels in the N9 microglial cell line, and inhibits LPS-induced NO production (Ferreira et al., 2010). In order to clarify whether Y_1R was involved on the inhibition of iNOS expression in retinal microglial cells, the retinal explants were treated with a Y_1R agonist (LP-NPY) and a selective Y_1R antagonist (BIBP3226). As previously reported in the N9 microglial cell line (Ferreira et al., 2010), LP-NPY significantly inhibited the LPS-induced increase in the immunoreactivity of iNOS in retinal microglia. The decrease in iNOS-IR could be possibly due to a decrease in the mRNA expression of iNOS, as observed by qRT-PCR. These results suggest that NPY could be preventing *de novo* synthesis of this enzyme via Y_1R activation. These observations were confirmed in primary mixed cultures of rat retinal neural cells. In these cultures iNOS-IR is mainly present in retinal microglial cells, and NPY also inhibited the increase of iNOS immunoreactivity. Again, using a pharmacological approach (using LP-NPY and BIBP3226), we have demonstrated that inhibition of iNOS-IR in microglial cells in these cultures occurs through Y_1R activation.

In the present work, we also identified an inhibitory role for NPY in LPS-induced ROS production in retinal microglial cells. Activated microglia are sources of reactive oxygen species that can cause neurotoxicity. It has been reported that superoxide production

precedes RGC apoptosis in a model of RGC degeneration (Kanamori et al., 2010). Our results show that superoxide production in cultured retinal explants exposed to LPS in mainly derived from microglial cells. However, trophic factors deprivation can also induce ROS production in photoreceptor cells in cultured retinal explants (Bhatt et al., 2010). Treatment of cultured retinal explants with NPY significantly decreased the production of superoxide by microglial cells. In accordance to our results, in another study, NPY inhibited the superoxide release from macrophages stimulated with zymosan (Dimitrijevic et al., 2005). Using cultures of purified retinal microglial cells, we confirmed that these cells are a major source of superoxide. Activated microglia can produce ROS through the stimulation of NADPH oxidase activity. This enzyme catalyzes the production of superoxide from oxygen, and it is inactive in resting phagocytes being activated by several stimuli, including LPS (Block et al., 2007). ROS are important for microglial functions, including survival and pro-inflammatory response. A dysregulation of ROS production in microglia may contribute to an overactivation, or even death, of microglial cells (Block et al., 2007). In this way, the decrease in the production of ROS might represent a protective mechanism in neurodegenerative diseases. However, once deleterious ROS levels have been reached, beneficial microglia apoptosis occurs. Thus, the same protective mechanism that inhibits ROS increase might also amplify microglia-mediated neurotoxicity.

As already mentioned, it is well known that microglia play an important role in mediating inflammatory processes in the CNS. In the retina, microglia-induced neurotoxicity can be triggered by LPS (Wang et al., 2005, Jiang et al., 2013) or retinal injury (Zhang et al., 2005b, Ng et al., 2009, Liu et al., 2012), resulting in the release of neurotoxic and pro-inflammatory factors, such as pro-inflammatory cytokines (Sappington and Calkins, 2006, Sivakumar et al., 2011, Chidlow et al., 2012). The state of activation of glial cells, the distribution of cytokine receptors and the downstream effector are important factors that determine the beneficial or deleterious role of cytokines on neurons (Figiel, 2008). Moreover, the role of cytokines can have different outcomes, depending on their concentration and exposure time. Interestingly, it has been reported that decreasing TNF-α concentration 10- to 100-fold can result in cell proliferation, instead of cell apoptosis in subventricular zone cultures (Bernardino et al., 2008). Moreover, even picogram concentrations of TNF-α (considered non-cytotoxic) can induce silencing of cell survival pathways (Venters et al., 2000). After exposure of cultured retinal explants to LPS, there was an increase in the mRNA expression of TNF-α, IL-1β and IL-6, as well as an increase in the protein levels of these cytokines in culture supernatants. Gene expression of TNF-α, IL-1β and IL-6 is mainly regulated by NF-κB. It has been demonstrated that NF-κB p65 nuclear

translocation is inhibited by NPY (Ferreira et al., 2010). However, when retinal explants were pre-treated with NPY, only mRNA levels of IL-1β were significantly decreased, an effect mimicked by Y_1R activation. In addition, after LPS challenge Y_1R agonist (LP-NPY) decreased the mRNA levels of TNF-α, an effect blocked by Y_1R antagonist (BIBP3226), showing that Y_1R activation mediates these protective effects, decreasing the mRNA levels of two pro-inflammatory cytokines (IL-1β and TNF-α). The discrepancy between NPY and Y_1R agonist effects could be due to the fact that NPY can act on other NPY receptors in addition to Y_1R, which could result in an antagonistic effect on TNF-α expression, similarly to what was previously described, where the suppressive effect on paw edema mediated by Y_1R activation, was opposed by the activation of Y_2R, which increased paw edema (Dimitrijevic et al., 2008).

Because it has been previously demonstrated that the expression of pro-inflammatory cytokines by activated microglia contributes to the pathology of various retinal degenerative diseases, including glaucoma (Yuan and Neufeld, 2000, Yoneda et al., 2001, Berger et al., 2008), we evaluated pro-inflammatory cytokine expression profile in a model of ischemia reperfusion injury. The results show that an increase in IOP to approximately 90 mmHg during 1 h did not induce a significant increase in the levels of TNF-α, IL-1β and IL-6 in the retina after 24 h of reperfusion. Microglial cells were described to be activated as early as 6 h after retinal ischemia (Zhang et al., 2005a). Moreover, other studies describe the expression peak of TNF-α (Husain et al., 2011), IL-1β (Yoneda et al., 2001) and IL-6 (Sanchez et al., 2003) in the retina between 4 and 12 h after reperfusion. Therefore, we hypothesized that 8 h after reperfusion changes in the inflammatory status of retinas would be better quantifiable. Retinal I/R injury induced an increase in the mRNA levels of TNF-α, IL-1β and IL-6 after 8 h of reperfusion. As a result of ischemic injury, resulting from increased IOP, glial cells can modify their gene expression profiles, potentially eliciting or aggravating neuronal damage, by the production and release of pro-inflammatory cytokines (Hangai et al., 1995, Tezel and Wax, 2000). The expression profiles of three such cytokines, TNF-α, IL-1β and IL-6 can follow different spatio-temporal patterns after retinal ischemic damage, and are dependent on the amount of increase in IOP comparing with normal IOP values (Yoneda et al., 2001, Sanchez et al., 2003, Berger et al., 2008, Dvoriantchikova et al., 2011, Husain et al., 2011). Interestingly, intravitreal injection of NPY decreased the mRNA expression of the three pro-inflammatory cytokines analyzed (TNF-α, IL-1β and IL-6) in the ischemic retinas 8 h after reperfusion, showing a potential role for NPY in inhibiting the pro-inflammatory responses after I/R injury. In other experimental model of glaucoma, laser photocoagulation induced microglia activation in the contralateral retinas (non-lasered) (Gallego et al., 2012).

In this work, the mRNA expression of IL-1β was possibly elevated in non-ischemic retinas also due to microglia activation in these retinas. Thus, NPY, in agreement with other work (De la Fuente et al., 2001), could be keeping the expression of this cytokine at optimal levels in the contralateral retinas.

Retinal I/R injury induced a robust increase in the production of TNF-α, IL-1β and IL-6, at 8 h after reperfusion, in good correlation with the levels of mRNA expression detected, and in contrast with what was found after 24 h of reperfusion. Cytokines such as TNF-α, IL-1β and IL-6, produced by microglia, can be used as markers of microglia shift to an activated phenotype in the retina, possibly mediating neuronal cell death (Schuetz and Thanos, 2004). NPY exhibited a potent inhibitory effect on IL-1β and IL-6 production in cultured retinal explants, which can be seen as a protective effect since these cytokines have been involved in many inflammatory processes in the retina (Kowluru and Odenbach, 2004, Mocan et al., 2006). In accordance to this observation, Ferreira and collaborators demonstrated that NPY can inhibit the release of IL-1β through suppression of NF-κB translocation to the nucleus in a microglia cell line (Ferreira et al., 2010). Moreover, the effect of NPY on IL-1β and IL-6 production was mimicked using an Y_1R agonist, and the inhibitory effect was blocked using a selective Y_1R antagonist (BIBP3226), indicating that Y_1R activation might mediate the protective effect of NPY. In opposition of what was reported previously in macrophages (De la Fuente et al., 2001), NPY did not inhibit the increase in TNF-α release triggered by LPS exposure. However, in the retinal I/R injury model, pre-treatment of retinas with NPY, injected intravitreally, inhibited the increase in TNF-α and also IL-6. The inhibition of TNF-α and IL-6 production can be interpreted as a protective modulation since these cytokines are involved in many pathological conditions and inflammatory processes. Moreover, a study describes that the blockade of TNF-α using the fusion protein Etanercept could reduce microglia activation and prevent the loss of RGCs in a model of glaucoma based on episcleral vein cauterization (Roh et al., 2012). Another study shows that retinal I/R induces an increase in TUNEL-positive cells in the INL and RGCL as early as 6 h after reperfusion, reaching a peak after 24 h (Chen et al., 2003), paralleling the temporal release of cytokines in this study. This could potentially indicate that an early therapeutic intervention, such as blocking TNF-α receptors or inhibiting microglia overactivation in retinal ischemia could possibly be more effective.

Ischemia is known to induce changes in the expression of NPY and NPY receptors in the rat hindlimb (Lee et al., 2003b). At physiological concentrations NPY stimulates neurogenic ischemic angiogenesis by activating Y_2R and Y_5R (Lee et al., 2003b). In this study, the mRNA expression of NPY and NPY receptors in the retina after ischemia followed by

24 h of reperfusion was not significantly different from the contralateral eye. In contrast, 8 h of reperfusion resulted in an increase in the mRNA expression of NPY and Y_2R, potentially indicating a role for NPY and Y_2R activation 8 h after I/R.

The role of NPY in the neuroinflammatory responses is not simple. Several reports describe opposite effects of NPY depending on the incubation time and concentration used, the cell type as well as on the type of stimulus. When incubated for a longer time period (20-30 min) NPY increases macrophage adhesion to tissue substrate, but has no effect with shorter times (10 min) of incubation (De la Fuente et al., 1993, De la Fuente et al., 2001). Using a range of lower concentrations of NPY (10^{-12}-10^{-8} M), NPY induces a stimulatory effect on the adherence, chemotaxis, phagocytosis and ROS production in macrophages (De la Fuente et al., 1993). In contrast, using higher concentrations of NPY (10^{-10}-10^{-5} M) there is an inhibition of macrophage migration after infection (Ahmed et al., 1998). Dimitrijević and colleagues reported that NPY increases NO production in LPS-stimulated macrophages (Dimitrijevic et al., 2008), while another study describes that NPY inhibits NO production in microglia exposed to LPS (Ferreira et al., 2010). Moreover, it has been demonstrated that the effect of NPY on leukocyte chemotaxis was abolished in the absence of adherent cells (Medina et al., 2000). Moreover, the effects of NPY on macrophage phagocytosis depend on the stimulus. In murine macrophages, the phagocytosis of latex beads was potentiated by NPY (De la Fuente et al., 2001). However, when the macrophages are first stimulated with zymosan, NPY inhibits phagocytosis (Dimitrijevic et al., 2005).

In summary, this group of results revealed that NPY is able to prevent microglia activation upon an endotoxin challenge and inhibits pro-inflammatory events in a model of retinal ischemia reperfusion injury. NPY inhibited morphological changes in microglial cells, iNOS expression, the production of ROS and the production and release of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. The results also demonstrate the involvement of Y_1R in the inhibition of retinal microglia activation and pro-inflammatory processes in the retina. This work revealed a novel role for NPY in modulating pro-inflammatory processes in the retina, especially controlling retinal microglia reactivity. Moreover, it demonstrates the involvement of Y_1R in the control of neuroinflammation in the retina. These findings may shed light in the discovery of new pharmacological approaches for the modulation of neuroinflammation in retinal degenerative diseases.

CONCLUSIONS

5. Conclusions

The results obtained in this work allowed drawing the following main conclusions:

In cultured retinal explants:

- Microglial cells express NPY and Y_1, Y_2 and Y_5 receptors;
- LPS decreases NPY and increases Y_1R mRNA expression;
- LPS induces morphological alterations in retinal microglia decreasing cell perimeter and Feret's maximum diameter, and increasing the circularity index, consistent with ameboid morphology;
- NPY inhibits alterations in the morphology of retinal microglial cells triggered by LPS, with Y_1R activation having an important role in this process;
- LPS increases the expression of inducible nitric oxide synthase (iNOS) in retinal microglial cells and this effect is blocked by NPY, involving Y_1R activation;
- LPS increases the production of reactive oxygen species (ROS) by microglial cells and NPY inhibits the increase in ROS production in retinal microglia;
- LPS increases the expression and production of TNF-α, IL-1β and IL-6;
- The increase in the expression and production of TNF-α triggered by LPS is not inhibited by NPY. However, NPY inhibits LPS-induced increase in IL-1β mRNA expression and IL-1β and IL-6 production;
- Y1R activation inhibits the increase in TNF-α and IL-1β mRNA expression and the production of TNF-α, IL-1β and IL-6 triggered by LPS.

In a model of retinal ischemia (60 min) and reperfusion (I/R) injury:

- Retinal microglial cells change to an ameboid shape after 24 h reperfusion;
- NPY and a Y_1R agonist (LP-NPY) inhibit the increase in the number of ameboid retinal microglia after I/R (24 h reperfusion);
- I/R injury upregulates the expression and production of TNF-α, IL-1β and IL-6 after 8 h, but not after 24 h reperfusion;
- Intravitreal injection of NPY before retinal ischemia decreases TNF-α, IL-1β and IL-6 mRNA expression and the production of TNF-α and IL-6 after 8 h reperfusion.

In conclusion, NPY is able to inhibit pro-inflammatory processes in the retina by preventing retinal microglia activation and the excessive production of several potential neurotoxic factors triggered by LPS in cultured retinal explants and by retinal I/R injury. NPY inhibits retinal microglia morphological changes to an ameboid shape, the increase in iNOS expression and in the production of potential neurotoxic factors such as ROS and pro-

inflammatory cytokines (TNF-α, IL-1β and IL-6). Moreover, this work also unveiled a predominant role for Y_1R in the prevention of pro-inflammatory responses in the retina.

The results obtained contributed to a better understanding of the role of the NPY system in neuroinflammation in the retina.

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