Université de Montréal

Role of Reactive Gliosis and Neuroinflammation in Experimental Glaucoma

Par

JORGE LUIS CUEVA VARGAS

Département de pathologie et biologie cellulaire

Faculté de Médecine

Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de Philosophie Doctor (Ph.D.)

en pathologie et biologie cellulaire

June 2015

Université de Montréal

Faculté des études supérieures et postdoctorales

Cette thèse de doctorat intitulée :

«Role of reactive gliosis and neuroinflammation in experimental glaucoma »

Présentée par:

JORGE LUIS CUEVA VARGAS

a été évaluée par un jury composé de personnes suivantes:

Hélène Girouard, PhD

président-rapporteur

Adriand Di Polo, Ph.D

directrice de recherche

Nathalie Arbour, PhD

Membre du jury

Marie-Ève Tremblay, PhD

examinateur externe

Graziella Di Cristo, PhD

représentant du doyen de la FES

RÉSUMÉ

Le glaucome est la principale cause de cécité irréversible dans le monde. Chez les patients atteints de cette pathologie, la perte de la vue résulte de la mort sélective des cellules ganglionnaires (CGR) de la rétine ainsi que de la dégénérescence axonale. La pression intraoculaire élevée est considérée le facteur de risque majeur pour le développement de cette maladie. Les thérapies actuelles emploient des traitements pharmacologiques et/ou chirurgicaux pour diminuer la pression oculaire. Néanmoins, la perte du champ visuel continue à progresser, impliquant des mécanismes indépendants de la pression intraoculaire dans la progression de la maladie. Il a été récemment démontré que des facteurs neuroinflammatoires pourraient être impliqués dans le développement du glaucome. Cette réponse est caractérisée par une régulation positive des cytokines pro-inflammatoires, en particulier du facteur de nécrose tumorale alpha (TNFα). Cependant, le mécanisme par lequel le processus neuroinflammatoire agit sur la mort neuronale reste à clarifier.

L'hypothèse principale de ce doctorat propose que les facteurs pro-inflammatoires comme le TNFα et la phosphodiestérase 4 (PDE4) interagissent avec les mécanismes moléculaires de la mort neuronale, favorisant ainsi la survie et la protection des CGRs au cours du glaucome.

Dans la première partie de ma thèse, J'ai utilisé un modèle *in vivo* de glaucome chez des rats Brown Norway pour montrer que l'expression du TNFα est augmentée après l'induction de l'hypertension oculaire. L'hypothèse spécifique de cette étude suggère que les niveaux élevés de TNFα provoquent la mort des CGRs en favorisant l'insertion de récepteurs AMPA perméables au calcium (CP-AMPAR) à la membrane cytoplasmique.

Pour tester cette hypothèse, j'ai utilisé un inhibiteur sélectif de la forme soluble du TNFα, le XPro1595. L'administration de cet agent pharmacologique a induit une protection significative des somas et des axones des neurones rétiniens. L'évaluation de la perméabilité au cobalt a montré que le TNFα soluble est impliqué dans l'insertion de CP-AMPAR à la membrane des CGRs lors du glaucome. L'exposition des neurones à une pression oculaire élevée est à l'origine de la hausse de la densité membranaire des CP-AMPARs, grâce à une diminution de l'expression de la sous-unité GluA2. La présence de GluA2 au sein du récepteur ne permet pas l'entrée du calcium à l'intérieur de la cellule. L'administration intraoculaire d'antagonistes spécifiques des CP-AMPARs promeut la protection des somas et des axones des CGRs. Ces résultats montrent que les CP-AMPARs jouent un rôle important dans la pathologie du glaucome.

Dans la deuxième partie de ma thèse, j'ai caractérisé l'effet neuroprotecteur d'un inhibiteur de la PDE4, l'ibudilast, dans notre modèle de glaucome. L'hypothèse spécifique s'oriente vers une atténuation de la réponse neuroinflammatoire et de la gliose par l'administration d'ibudilast, favorisant ainsi la protection neuronale. Les résultats montrent que dans les rétines glaucomateuses, l'ibudilast diminue la gliose et l'expression de plusieurs facteurs tels que le TNFα, l'interleukine-1β (IL-1β), l'interleukine-6 (IL-6) et le facteur inhibiteur de la migration des macrophages (MIF). Chez les rats glaucomateux, nous avons observé une expression notable de PDE4A dans les cellules de Müller, qui est en corrélation avec l'accumulation de l'AMP cyclique (AMPc) dans ces cellules après un traitement d'ibudilast. Finalement, nous avons démontré que la protection des CGRs via l'administration d'ibudilast est un mécanisme dépendent de l'AMPc et de la protéine kinase A (PKA).

En conclusion, les résultats présentés dans cette thèse identifient deux mécanismes différents impliqués dans la perte des CGRs au cours du glaucome. Ces mécanismes pourraient fournir des perspectives potentielles pour le développement de nouvelles stratégies de traitement du glaucome.

Mot clés : cellules ganglionnaires de la rétine, glaucome, facteur de nécrose tumorale alpha, neuro-inflammation, ibudilast, phosphodiestérase-4, adénosine monophosphate cyclique, CP-AMPAR

SUMMARY

Glaucoma is the leading cause of irreversible blindness worldwide. Loss of vision in glaucoma results from the selective death of retinal ganglion cells (RGCs) and axonal degeneration. Elevated intraocular pressure (IOP) is the major risk factor for developing glaucoma, and current therapies have focused on pharmacological or surgical strategies to lower IOP. However, visual field loss continues to progress in spite of effective pressure control, indicating that mechanisms other than elevated IOP contribute to disease progression. Recent data demonstrate a neuroinflammatory component in glaucoma, characterized by upregulation of proinflammatory cytokines, most notably tumor necrosis factor α (TNFα). However, the mechanism by which the neuroinflammatory response acts on RGC death needs to be clarified.

The main hypothesis of this thesis is that targeting pro-inflammatory factors including TNFα and phosphodiesterase-type 4 (PDE4), interferes with molecular mechanisms that contribute to RGC death and this will thus successfully promote neuronal protection.

In the first part of my thesis, I used an *in vivo* glaucoma model in Brown Norway rats to show that TNFα is upregulated early after induction of ocular hypertension. The specific hypothesis of this study is that high levels of TNFα promote RGC death by mediating the membrane insertion of Ca^{2+}-permeable AMPA receptors (CP-AMPARs). I blocked TNFα function with XPro1595, a selective inhibitor of soluble TNFα. Administration of XPro1595 effectively protected RGC soma and axons. The cobalt permeability assay was used to show that soluble TNFα triggers the membrane insertion of CP-AMPAR in RGCs of glaucomatous retinas. This CP-AMPAR activation is caused by the downregulation of GluA2 which occurs when neurons are exposed to elevated IOP. Finally, intraocular

administration of specific CP-AMPAR antagonists promoted RGC soma and axon protection. Taken together, these results show that CP-AMPARs play an important role in in the pathology of glaucoma.

In the second part of my thesis, I characterized the neuroprotective effect of ibudilast, an inhibitor of PDE4, in the Brown Norway glaucoma model. We hypothesized that ibudilast promotes neuron protection by attenuating gliosis and the neuroinflammatory response. The results show that in glaucomatous retinas, ibudilast attenuates gliosis and the expression of TNFα, interleukin-1β (IL-1β), interleukin-6 (IL-6) and macrophage migration inhibitory factor (MIF). Interestingly, elevated IOP leads to substantial expression of PDE4A in Müller cells, which correlates with the accumulation of cAMP in these cells after ibudilast treatment. Lastly, ibudilast promoted RGC soma and axons protection through the activation of the cAMP/PKA pathway.

In conclusion, the findings presented in this thesis identify two different mechanisms underlying RGC loss in glaucoma. These mechanisms can potentially provide new insights to develop novel strategies for the treatment of glaucoma.

Key words: retinal ganglion cell, glaucoma, tumor necrosis factor-alpha, neuroinflammation, ibudilast, phosphodiesterase-4, cyclic adenosine monophosphate

TABLE OF CONTENTS

LIST OF TABLES.

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ADAR	adenosine deaminase acting on RNA
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	ativated protein 1
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
cFLIP	caspase FLICE-like inhibitory protein
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CNTF	cilliary neurotrophic factor
CRALBP	cellular retinaldehyde-binding protein
CREB	cAMP response element binding protein
EAE	experimental autoimmune encephalomyelitis
EPSC	excitatory post-synaptic current
ERK	extracellular signal-regulated kinases
ET-1	endothelin-1
FADD	fas-associated protein with death domain
GABA	gamma-aminobutyric acid
GCL	ganglion cell layer
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein

GLAST	glutamate-aspartate transporter
GPNMB	Transmembrane glycoprotein NMB
GS	glutamine synthetase
HSP	heat shock protein
IAP	inhibitor of apoptosis protein
IKK	IκB kinase
IL-1β	interleukin-1β
IL-6	interleukin-6
INL	inner nuclear layer
iNOS	inducible NO synthase
IOP	intraocular pressure
IPL	inner plexiform layer
JAK/STAT	janus kinase/signal transducers and activators of transcription
LGN	lateral geniculate nucleu
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MIF	migration macrophage inhibitory factor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFL	nerve fiber layer
NMDA	N-methyl-D-aspartate receptor
NO	nitric oxide
NSF	*N*-ethylmaleimide-sensitive fusion protein
NTG	normal tension glaucoma

OHT	ocular hypertension
ONH	optic nerve head
ONL	outer nuclear layer
OPL	outer plexiform layer
PACG	primary angle closure glaucoma
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PEDF	pigment epithelium-derived factor
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PICK1	protein interacting with C kinase 1
PKA	Protein kinase A
PKCα	protein kinase C alpha
POAG	primary open-angle glaucoma
PRR	pattern recognition receptor
RBPMS	RNA-binding protein with multiple splicing
REST	repressor element-1 silencing transcription factor
RGC	retinal ganglion cell
RIP	receptor-interacting protein
siRNA	small interfering RNA
TACE	tumor necrosis factor-alpha converting enzyme
TLR	toll-like receptor
TNFα	tumor necrosis factor-alpha

TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TRADD	TNFR1 associated death domain protein
TRAF2	TNF receptor-associated factor 2
TrkB	tropomyosin receptor kinase B
TRPV4	transient receptor potential cation channel, subfamily V, member 4
TSPO	Translocator protein
TYRP1	Tyrosinase-related protein 1

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor Dr. Adriana Di Polo for the opportunity she gave me to be involved in her research. I am deeply indebted to her for the guidance she has given me over the past several years. Her scientific vision and thoughtful criticism will help me to be a competent and productive professional.

I would also like to thank our collaborators Dr. Philip Barker and Dr. Derek Bowie, from McGill University for their expertise and support to the first article in this thesis.

I am grateful to my thesis jury members Dr. Hélène Girouard, Dr. Marie-Ève Tremblay, Dr. Nathalie Arbour, and Dr. Graziella Di Cristo for accepting to evaluate my work. I also appreciate the comments and encouragement from my advisory committee members Dr. Nicole Lecrerc and Dr. Elvire Vaucher.

Working in the lab would not have been amazing without the presence of great people: Sri, Ali, Dara, Ariel, Barbara, Nicolas, Yoko, Jessica, Marius, Florence and Pegah. Writing these lines, many special memories came to my head. Thanks to all of you. You certainly made this experience much more exciting and memorable. Ingrid, Nicolas Unsain, and Joseph III, discussing with you it was always a pleasure.

I wish to appreciate the availability of Dr. Nathalie and Laurine during FACS experiments.

I would like to thank my wife, my parents and my brother for encouraging me to pursue my scientific interest and supporting me especially during the difficult and stressful time.

To my parents, Aurea and Eudomaro, for their dedication, help and understanding

To my brother, Jose Alberto, for his encouragement

To my wife, Heidy, for her patience and priceless support

To my son, Marcelo, for his future

To Miss Adriana, wherever she is, for her support.

CHAPTER 1

I. GENERAL INTRODUCTION

I.1. THE RETINA: CELLULAR AND FUNCTIONAL ORGANIZATION

The retina is a specialized light-sensitive tissue comprised of neurons and glial cells that captures photons of light and sends this information to the brain for further processing. The neurons are sub-divided into photoreceptors cells, bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells (RGC). The glial cells are categorized into Müller cells, astrocytes and microglia. These cells are organized into five interconnected layers. Of the five layers, the ganglion cell layers (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) consist mainly of cell bodies while synaptic connections among the neurons are located in the inner plexiform layer (IPL) and the outer plexiform layer (OPL) (Fig. 1). Briefly, the light pathway is as follows: light passing through the lens is captured by the photoreceptors, initiating and sending a cascade of neuronal signals which is eventually sent to the brain via RGCs, where it is further processed for visual perception (Kolb, 1995a).

The retina is an extension of the central nervous system (CNS) and, as such, is considered a window to the brain. In fact, the retina displays many structural similarities to the brain and spinal cord. Moreover, a multitude of extremely similar pathological processes contribute to neurodegenerative diseases affecting the brain, spinal cord and eye (London et al., 2013). For example, there is much evidence that supports a link between Alzheimer's disease and glaucoma. Patients with Alzheimer's disease show an increased risk of having glaucoma (Bayer et al., 2002), also, some Alzheimer patients exhibit optic nerve degeneration (Hinton et al., 1986) and loss of RGCs (Blanks et al., 1989). Moreover, amyloid plaques and hyperphosphorylated tau protein have been reported in post-mortem retinas from patients with Alzheimer's disease. Recently, it has been reported that tau has a neurotoxic effect in a model of experimental glaucoma (Chiasseu M, 2015). Further understanding the similarities among

CNS regions and neurodegenerative conditions is essential for the development of shared therapies. One pertinent example is the use of galantamine, which is currently used for the symptomatic treatment of Alzheimer's disease (Razay and Wilcock, 2008). Interestingly, galantamine has also been shown to be involved in RGC protection in a rodent model of glaucoma disease (Almasieh et al., 2010), and thus represents a promising treatment option for human glaucomatous patients.

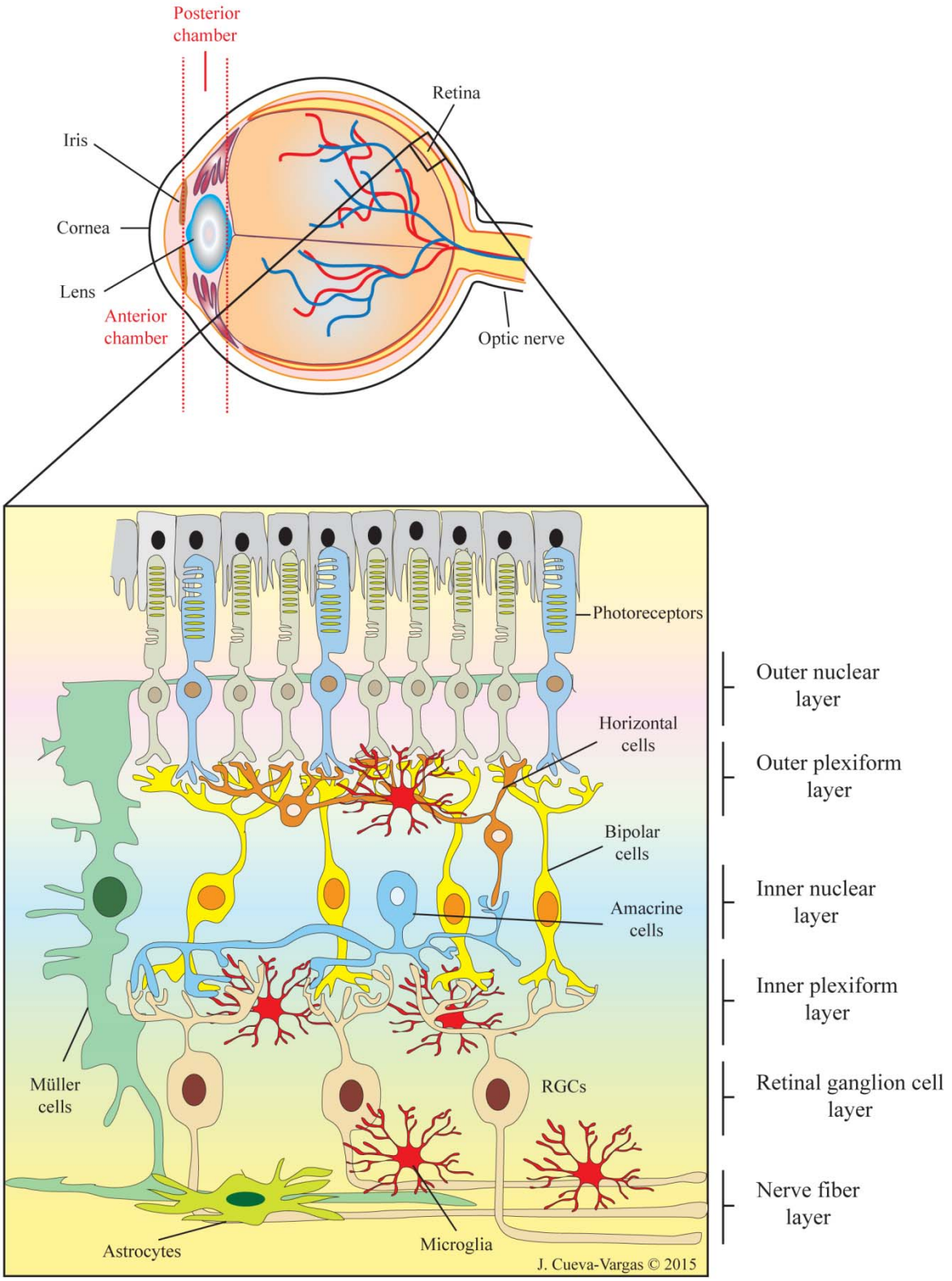

Posterior chamber
Retina
Iris
Cornea
Lens
Anterior chamber
Optic nerve
Photoreceptors
Horizontal cells
Bipolar cells
Amacrine cells
Müller cells
RGCs
Astrocytes
Microglia
J. Cueva-Vargas © 2015
Outer nuclear layer
Outer plexiform layer
Inner nuclear layer
Inner plexiform layer
Retinal ganglion cell layer
Nerve fiber layer

Figure 1. A schematic diagram showing the glial cells and neurons in the retina.

The light from the lens traverse all retinal layers before reaching the primary light-sensitive cells called photoreceptors (rods and cones). Photoreceptors transduce light stimuli into electrical signal. Rods are able to detect even a single photon and cones are responsible for vision at higher intensity illumination and color vision. The signal is then transferred to the bipolar cells and horizontal cells at the level of the OPL, where the synaptic connections are achieved. Bipolar cells and amacrine cells make synaptic connection with RGC in the IPL. RGC axons convey the information from the retina to the brain via de optic nerve. Glial cells include: Müller cells, astrocytes and microglia. They contribute to create a favourable environment for neurons. Source of image: Jorge Luis Cueva Vargas.

I.1.1. Retinal neurons

More than 60 distinct types of neurons are needed for the processing of visual images. They are broadly grouped into sensory neurons, including the photoreceptors, and other intricately connected neurons, which propagate the signal for image processing to occur. The connected neurons are classified based on the specific role in processing visual images and are arranged in three categories: 1) those which decompose the output from photoreceptors, 2) those which connect the outputs from photoreceptor to specific types of RGCs and, 3) those which combine activities from bipolar and amacrine cells to create the different signals encoding the visual world (Masland, 2011, 2012b).

I.1.1.1. Photoreceptors

The cell bodies of the photoreceptors are located in the ONL. At the OPL, the axon terminals of photoreceptors make synaptic contact with bipolar and horizontal cells. Through the process of phototransduction, photoreceptors convert captured light energy into membrane potential changes by altering the release of neurotransmitters (Cuenca et al., 2014). Photoreceptors are categorized into rods and cones. Rods contain a light-sensitive pigment known as rhodopsin. Although rods cannot differentiate between spectral modulation, rods are highly light-sensitive and are able to detect even a single photon (Rieke, 2000) and are thus responsible for dim-light vision (Hoon et al., 2014). On the other hand, cones-opsins, are less sensitive than rods. They respond selectively to photons in different regions of the visible spectrum. There are three types of cones: long (red or L-), middle (green or M-) and short (blue or S-) wavelength cones (Wassle, 2004), thus conferring the ability to visualize the entire color pallete.

I.1.1.2. Bipolar cells

Bipolar cells are located in the INL and they transmit signal from the photoreceptors to the inner retina. Recent studies indicate that there are 13 bipolar cell sub-types (Helmstaedter et al., 2013; Euler et al., 2014). In mice, there is one type of rod bipolar cell, whose dendrites exclusively contact rod photoreceptors; and 12 types of cones bipolar cells that make synaptic contact with cones and in some species can also contact rods (Nelson and Connaughton, 1995). When the retina is stimulated by light, the OFF bipolar cells hyperpolarize and the ON bipolar cells depolarize. OFF bipolar cells express ionotropic glutamate receptors and make contact with OFF RGCs, whereas ON bipolar cells express metabotropic glutamate receptors and make contact with ON RGCs (Nomura et al., 1994; Wassle, 2004).

I.1.1.3. Horizontal cells

Similar to bipolar cells, horizontal cells are located in the INL. These cells release γ-aminobutyric acid (GABA) and provide inhibitory feedback to the photoreceptors through lateral interactions in the OPL. Some reports suggest that light-dependent release of GABA from horizontal cells provide feed-forward inhibition of bipolar cell dendrites (Wassle, 2004). Horizontal cells measure the average level of illumination across the broad region, then subtract it from the local signals triggered by the photoreceptors, resulting in the reduction of redundancy of the signal transmitted to the inner retina (Masland, 2001).

I.1.1.4. Amacrine cells

Amacrine cells are the main group of inhibitory interneurons interacting with bipolar, ganglion cells and other amacrine cells in the IPL (Masland, 2012a). They exhibit great structural diversity and complexity. Indeed, they serve to integrate, modulate and interpose a

temporal domain to the visual message presented to the RGCs (Kolb, 1995b). Wild-field amacrine cells mediate segregation of object and background motion (Olveczky et al., 2003).

I.1.1.5. Retinal ganglion cells

There are approximately 20 sub-types of RGCs in the retina, differentiated by morphology, molecular characteristics and functionality (Erskine and Herrera, 2014). RGCs have a unique configuration and can be structurally compartmentalized in dendrites, cell body, non-myelinated axon and myelinated axon (Yu et al., 2013). In the IPL, RGC dendrites receive the electrical messages from bipolar cells and/or amacrine cells. Through the RGC axons, these messages are conveyed to specific targets in the brain. RGC axons travel through the optic nerve, the chiasm and the optic tract to make synapses in the superior colliculi, the lateral and medial geniculate bodies and the pretectal nuclei. Interestingly, it has been reported that 1-3% of the RGCs population contain melanopsin. This RGCs population, which is inherently light-sensitive is involved in the circadian rhythm and the pupillary light reflex (Wassle, 2004)

I.1.1.5.1. Retinal ganglion cells: the cellular target of many optic neuropathies

Optic neuropathies are a group of diseases characterized by dysfunction and/or degeneration of axons in the optic nerve and RGC death, leading to vision loss. Each RGC compartment is located in a different extracellular environment with different energy requirement (Yu et al., 2013). With the exception of the myelinated axons, the energy demand in the other compartments is higher, making RGCs more vulnerable to injury (Morgan, 2012). Non-myelinated axons have a high density of mitochondria-rich varicosities (associated with a high demand of energy) and a limited blood supply (associated with low energy delivery).

This imbalance of energy supply and demand, combined with the fact that action potentials are conducted in a non-saltatory fashion (the action potential travels the full length of the axon in the absence of Ranvier's node), makes non-myelinated axons more vulnerable to ischemic injury. Also, the RGC axons in the optic nerve head (ONH) appear to be highly vulnerable to injury. Indeed, the ONH is considered to be the primary site of axonal injury in glaucoma (Chidlow et al., 2011). Axonal injury has deleterious effect on axonal cytoskeleton and axonal transport (Yu et al., 2013) and also affects ONH structure including astrocytes (Hernandez et al., 2008), levels of nitric oxide synthase (Neufeld et al., 1997) and mitochondrial cytochrome c oxydase expression (Balaratnasingam et al., 2009). In fact, axonal injury promotes a decrease of glutathione leading to oxidative stress in glial cells and neurons (Hernandez et al., 2008). In addition, some studies have demonstrated that maintaining axonal integrity is vital to enhance neuronal survival and prevent glial reactivity (Yu et al., 2013), Thus, the axonal compartment plays an important role in RGC injury.

I.1.2. Glial cells

As previously mentioned, the retina's main goal is to convey light information captured by photoreceptors to the brain for further processing. To achieve this function, neurons need an appropriate and favourable environment, which is supported by glial cells, the non-neuronal cells that also play a large role in damage repair in the retina. In this section I will discuss the role of glial cells in the healthy retina.

I.1.2.1. Müller cells

Müller cells are considered to be the major glial cell type in the retina, spanning the entire thickness of the tissue. The somata of Müller cells are located in the INL, with two

processes extending in opposite directions. Müller cells processes and soma extend side branches, allowing the cell to be in close contact with, and in many cases ensheath, virtually all neuronal elements as well as the blood vessels in the retina (Bringmann et al., 2006). In the healthy retina, Müller cells are active contributors to the retinal structure and function. They create a favorable environment by regulating the concentration of neurotransmitters, including glutamate and GABA. The clearance of glutamate, mainly via glutamate-aspartate transporter (GLAST), is important to prevent neurotoxicity (Reichenbach and Bringmann, 2013). In Müller cells, glutamine synthetase (GS) converts glutamate to glutamine. Glutamine is then taken up by neurons as a precursor for the synthesis of glutamate and GABA (Pow and Crook, 1996). Several studies have reported that Müller cells protect retinal neurons, particularly cones, by secreting neurotrophic factors, growth factors and cytokines. Also, Müller cells phagocytose debris from dead neurons or pigment epithelial cells (Francke et al., 2001; Bringmann et al., 2009). In addition, it has been shown that these cells, and not astrocytes, act as living fibers by guiding the light through the inner retina towards the photoreceptors (Franze et al., 2007). Another very important role of Müller cell is the control of osmotic and ionic homeostasis through the actions of the glial water channel aquaporin-4 (AQP4) and the potassium-dependent Kir4.1 channels, respectively (Nagelhus et al., 1999). In addition, Müller cells maintain the inner blood-retinal barrier and provide a permanent anti-proliferative environment for retinal vascular endothelial cells, by secreting pigment epithelium-derived factor (PDEF), thrombospondin-1 and glial cell-line derived neurotrophic factors (GDNF) (Eichler et al., 2004; Nishikiori et al., 2007). During the last decade, it has been found that Müller cells regulate synaptic transmission by releasing excitatory gliotransmitters such as glutamate and adenosine triphosphate (ATP) (Newman, 2004) as well as inhibitory

gliotransmitters such as adenosine (Housley et al., 2009), These gliotransmitters act on retinal neurons and on Müller cells themselves by mediating the propagation of intracellular calcium waves. Mechanosensory Ca^{2+} ion channels, such as TRPV4 have been reported in Müller cells, and not in retinal astrocytes, to provide the retina with biomechanical sensors (Ryskamp et al., 2011; Ryskamp et al., 2014) Interestingly, it has been recently shown that, based on their gene profile, some mature Müller cells can generate neural stem cells (Das et al., 2006; Bernardos et al., 2007). The ability of Müller cells to dedifferentiate makes them an interesting cell-type to study in the development of regenerative therapies in retinal neurodegenerative diseases.

I.1.2.2. Astrocytes

Astrocytes originate from the optic nerve and then migrate to the nerve fiber layer (NFL) (Watanabe and Raff, 1988). In the retina, astrocytes share some functions with Müller cells. Indeed, astrocytes are involved in the development and function of the retinal vasculature, blood flow and maintenance of the blood-retinal barrier (Zhang and Stone, 1997; Kur et al., 2012). In addition, astrocytes play an important role in the maintenance of the glutamate homeostasis and clearance (Kimelberg, 2010), and modulation of synaptic transmission (Newman, 2004). In the non-myelinated ONH, astrocytes are the most abundant glial cells, supporting the function of the axons. Astrocytes supply energy substrates to the axons and maintain the extracellular pH and ion homeostasis in the periaxonal space (Hernandez et al., 2008).

I.1.2.3. Microglia

Microglia are specialized mesodermal/mesenchymal-derived cells with immunological properties in the CNS (Chan et al., 2007). Recently, genetic-fate mapping essays have demonstrated that microglia are derived from the yolk sac and the monocytes are derived from haematopoiesis in the bone marrow (Ginhoux et al., 2010; Kierdorf et al., 2013; Prinz and Priller, 2014). In the mature healthy retina, ramified resting microglia form a dynamic and highly organized non-overlapping network. This configuration allows the microglia to survey the microenvironment and participate in cell-cell interactions with retinal neurons and other macroglia (Karlstetter et al., 2014). In fact, the presence of surface proteins such as receptors for cytokines, growth factors, purinergic ligands, toll like receptors (TLR), chemokines, and complement components allows microglia to systematically sense their environment. Microglial activation is a highly regulated process. Several inhibitory mechanisms regulate microglia activation. For example, the transmembrane glycoprotein CD200, expressed in many different retinal cell types serves as a ligand for CD200R, which is the CD200 cognate receptor expressed by microglia. The physical interaction between CD200 and CD200R blocks pro-inflammatory activation of microglia. In fact, CD200-deficient mice have a highly density of microglia and an early onset of experimental autoimmune encephalomyelitis (EAE) (Hoek et al., 2000; Wright et al., 2000). In addition, blockade of CD200R increases the inflammatory response in the retina and aggravates experimental autoimmune uveoretinitis (Banerjee and Dick, 2004). Another well-known example is the binding of CX3CL1 to CX3CR1, which prevents neurotoxicity, by attenuating microglia reactivity (Cardona et al., 2006). Finally, microglia also interact with Müller cells to maintain an optimal level of neurotrophic factors necessary for the survival of neurons. Thus, microglia can produce or

trigger the release of several neurotrophic factors including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and basic fibroblast growth factor (bFGF) from Müller cells. These neurotrophic factors, which are secreted following injury and light induced degeneration, are thought to support photoreceptor survival (Harada et al., 2002; Harada et al., 2003; Langmann, 2007)

I.2. Glaucoma

I.2.1. Definition, epidemiology and risk factors

The term "glaucoma" represents a group of optic neuropathies in which progressive visual field loss is caused by the cupping of the ONH (damaged nerve accompanied by an enlarged central cup and thin rim) and the loss of RGCs and their axons. Glaucoma is one of the leading causes of blindness worldwide. At the present time, more than 64 million people are affected by the disease. The number of affected people is expected to increase to 76 million by 2020 and 111.8 million by 2040 (Quigley and Broman, 2006; Tham et al., 2014).

Despite many studies examining the cause of glaucoma, the etiology of this disease is still unknown. However, there are several risks factors associated with increased likelihood of developing glaucoma. Among these factors elevated intraocular pressure (IOP) is the primary risk factor to develop glaucomatous damage (Gordon et al., 2002) as well as for the progression of the disease (Anderson and Normal Tension Glaucoma, 2003). Interestingly, it has been reported that in patients with high IOP, thin central corneal thickness is also considered an additional risk factor for developing the disease (European Glaucoma Prevention Study et al., 2007; Manni et al., 2008). Although elevated IOP can be lowered

either surgically or using pharmacological treatment, visual field loss continues to progress in many glaucoma patients. Indeed, approximately 50% of glaucoma patients have IOP-independent mechanisms of optic nerve damage (Giangiacomo, 2009). Increasing age is another critical risk factor for the development of glaucoma and for patients who already suffer from ocular hypertension (OHT). In fact, individuals aged 40 years or older are at a much higher risk of developing glaucoma than other age groups (Coleman and Miglior, 2008). Older age is considered as a surrogate risk factor for many biological processes associated with aging. For example, age-related increase in oxidative damage and autophagy promotes dysfunction of the trabecular meshwork, thereby resulting in elevated IOP (Sacca and Izzotti, 2008; Pulliero et al., 2014). Several others factors that have been suggested to increase the risk of developing glaucoma including: myopia (Xu et al., 2007), family history (Klein et al., 2004), hypertension (Mitchell et al., 2004) and ethnicity (Leske et al., 1994; Boland and Quigley, 2007).

I.2.2. Types of glaucoma

Although damage of RGC axons is a common feature of all forms of glaucoma, different pathophysiological mechanisms contribute to the disease. Here, I will discuss the two most common types of glaucoma associated with IOP elevation: Primary Open-Angle Glaucoma (POAG) and Primary Angle-Closure Glaucoma (PACG). Both POAG and PACG are generated by increased resistance to aqueous humor drainage. Aqueous humor is produced by the epithelium of the ciliary body. Filling the posterior and anterior chambers, aqueous humor provides nutrients to the lens, cornea and retina. In addition, aqueous humor removes metabolic waste as it flows from the posterior chamber through the pupil to the anterior chamber where it exits the eye via the trabecular meshwork (Kiel et al., 2011). In the

iridocorneal angle, the aqueous humor is drained from the eye by passing through the trabecular meshwork and the inner wall of Schlemm's canal, eventually collecting into the episcleral veins (Goel et al., 2010) (Fig. 2A). Lastly, I will discuss normal tension glaucoma (NTG), which occurs independently of resistance to aqueous humor drainage.

I.2.2.1. Primary Open-Angle Glaucoma

POAG is the most common type of glaucoma with a prevalence of 3.05% and a particularly high incidence in people of African ancestry. It has been estimated that there will be 52.7 million of cases by the year 2020 (Tham et al., 2014). POAG is characterized by changes at the level of the optic disc, atrophy of the RGC axons in the optic nerve and loss of RGCs in the retina, which results in progressive loss of visual field (Quigley, 2005). Elevated IOP is a major risk factor for developing POAG. Unlike other forms of glaucoma, this increase in IOP is not caused by any anatomical obstruction in the aqueous humor flow pathway. (Fig.2B). Instead, the increase in IOP is caused by resistance to the aqueous humor outflow, which has been associated with age-related and/or pathological changes in the trabecular meshwork or Schlemm's channel. Indeed, the presence of free radicals, the main factor contributing to cell aging, has been reported to affect directly trabecular meshwork cells (Wordinger and Clark, 1999; Gabelt and Kaufman, 2005; Sacca and Izzotti, 2008; Pulliero et al., 2014).

I.2.2.2. Primary Angle-Closure Glaucoma

The prevalence of PACG is 0.50% with high incidence occurring primarily in Asian. It has been estimated that 23.4 million of people will have PACG by 2020 (Tham et al., 2014). Even though there are less cases of PACG relative to POAG, more PACG patients become

blind. PACG is characterized by the resistance of the aqueous humor flow through the pupil caused by abnormal anatomy of the iris including pupillary blockage and angle crowding (Fig. 2C). Both mechanisms often co-exist to trigger OHT (Quigley et al., 2003; Tarongoy et al., 2009). Pupillary blockage occurs when the iris-lens channel impedes the normal flow of aqueous humor from the posterior chamber to the anterior chamber. As a result, the pressure increases in the posterior chamber and pushes the periphery of the iris forward resulting in closure of the iridocorneal angle. Angle crowding occurs when the peripheral iris and the TM are in contact with each other, thereby impeding the drainage of aqueous humor from the trabecular meshwork.

I.2.2.3. Normal Tension Glaucoma

NTG is considered a subtype of POAG. NTG patients present with an unobstructed iridocorneal angle and IOP considered to be in the normal range. Several studies have shown that 40-75% of people diagnosed with POAG have NTG (Desai and Caprioli, 2008). The visual field loss in NTG patient progresses with a similar rate as POAG patients. Although the cause of NTG is currently unknown, decreased blood flow at the optic disc has been associated with the development of NTG (Sato et al., 2006). Vascular dysfunction has been confirmed by magnetic resonance imaging studies in NTG patients. The over-expression of endothelin-1 (ET-1) in vascular endothelial cells has also been associated with the development of NTG. Application of ET-1 is used to induce ischemic optic neuropathy in different animals such as monkeys, rabbits, and rats (Mi et al., 2014). Another IOP-independent mechanism that has been reported is the elevated expression of antibodies against heat shock proteins (HSPs) in the sera of NTG patients (Wax et al., 1994; Wax et al., 1998), suggesting an immunogenic factor underlying the pathology of NTG.

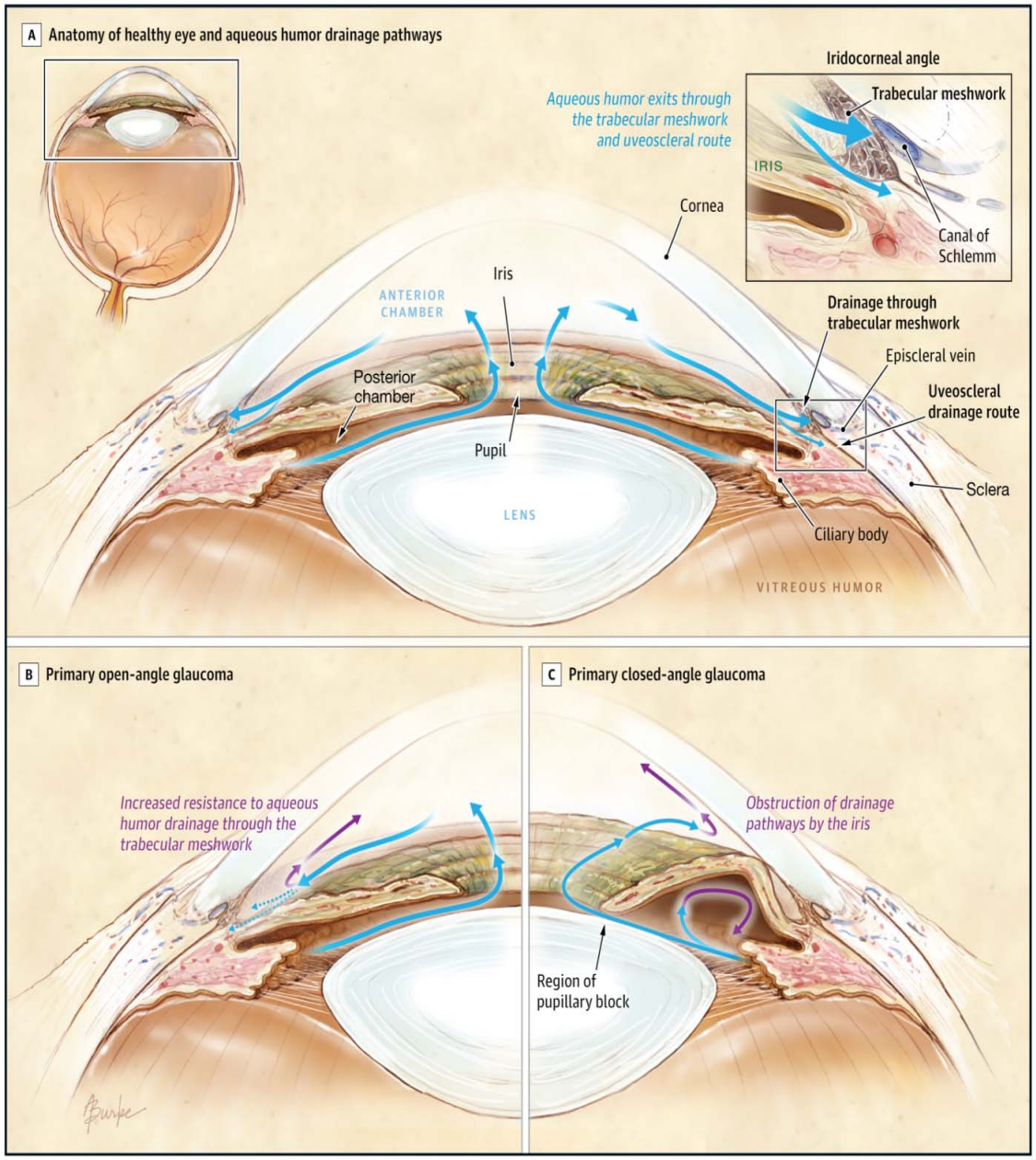

A Anatomy of healthy eye and aqueous humor drainage pathways
Iridocorneal angle
Trabecular meshwork
IRIS
Canal of Schlemm
Aqueous humor exits through the trabecular meshwork and uveoscleral route
Cornea
Iris
ANTERIOR CHAMBER
Drainage through trabecular meshwork
Episcleral vein
Posterior chamber
Uveoscleral drainage route
Pupil
Sclera
LENS
Ciliary body
VITREOUS HUMOR
B Primary open-angle glaucoma
Increased resistance to aqueous humor drainage through the trabecular meshwork
C Primary closed-angle glaucoma
Obstruction of drainage pathways by the iris
Region of pupillary block

Figure 2. A schematic presentation of the structures involved in the aqueous humor circulation within the eye in healthy and glaucomatous eyes. A. The vitreous humor is produced by the ciliary body in the posterior chamber and enters the anterior chamber through the pupil bringing nutrients to the lens, iris and cornea. Subsequently, the aqueous humor is drained at the trabecular meshwork through the Schlemm's canal. **B**. In primary open-angle glaucoma (POAG) there is an increased resistance to aqueous humor outflow at the level of trabecular meshwork. These resistance creates elevated intraocular pressure (IOP). **C.** In primary angle-closed glaucoma (PACG), there is a physical closure due to anatomical abnormalities such as pupillary block and angle crowding. These modifications are present together or individually creating also a resitance to the aqueous humor drainaged. Source: (Weinreb et al., 2014) The Pathophysiology and Treatment of Glaucoma: A Review. Copyright © American Medical Association.

I.2.3. Models of experimental glaucoma: advantages and disadvantages

Without a doubt, animal models have improved our understanding of a variety of mechanisms that cause human disease. Animal models are also a useful tool to discover new targets for therapeutic drugs and the processes by which these drugs act. Many models of spontaneous and induced OHT have been used to study glaucoma. These models have provided us with new information on the cellular and molecular mechanisms concerning the pathology of RGCs death and axon degeneration. In this section, I will discuss the most relevant models of experimental glaucoma currently available for research purposes.

I.2.3.1 Non-human primate models

In Puerto Rico, a naturally-occurring group of rhesus monkeys with high tension POAG were discovered. These animals show RGC death, atrophy of the optic nerve, and loss of visual field (Dawson et al., 1993). OHT has also been induced in non-human primates. For example, argon laser photocoagulation results in successful IOP elevation in 70% of induced monkeys. In these monkeys, histopathological analysis shows loss of RGCs, ONH cupping, and thinning of the NFL (Gaasterland and Kupfer, 1974). OHT in non-human primates was also achieved by using latex microspheres (Weber and Zelenak, 2001) and ghost of red blood cells (RBC) that consist of RBC which lave lost much or all of their hemoglobin, conferring to the cells rigidity (Quigley and Addicks, 1980). The close phylogeny and anatomical similarities between the optic nerve of humans and non-human primates make monkeys an excellent model for studying glaucoma. However, the high cost of purchasing and housing monkeys together with their limited availability are major disadvantages, particularly for

neuroprotective studies where large number of animals are necessary. Another disadvantage is the need for experienced personnel to handle these animals.

I.2.3.2. Rodent models of glaucoma

Rodent models are a good alternative to non-human primate models. Rodents are relatively inexpensive to purchase and maintain, allowing for the use of more animals per experiment. Another advantage is that they are also easy to handle. In fact, we are able to accurately measure IOP in awake animals, without the use of anesthetics. However, one major limitation of rodent models is the presence of important anatomical differences with respect to primates. For example, unlike humans, the rat ONH does not have a well-developed lamina cribrosa. Lamina cribrosa in primates is a structure comprised of approximately 10 plates of connective tissue through which the axons travel. These plates are composed by laminin, elastine, collagens, and provide a substrate for different cell types including astrocytes and lamina cribrosa cells (Morrison et al., 2005). In rodents, there is not lamina cribrosa. The rat laninar region has sparse collagenous beams containing elastin fibrils and is called glial lamina (Howell et al., 2007). Nonetheless, there are several similarities between the human and rat ocular tissues including the presence of astrocytes and unmyelinated axons in the ONH (Morrison et al., 1995), and the anterior chamber structures that are important for proper aqueous humor outflow to occur (van der Zypen, 1977). Here, I will first discuss the inducible rodent models of glaucoma, followed by the genetic rodent models of glaucoma.

I.2.3.2.1. Hypertonic saline injection

In this model, a hypertonic saline solution (NaCl 1.8 M) is injected into the episcleral vein of rats. The injection of the saline solution causes cell membrane damage and scarring in

the trabecular meshwork. Therefore, IOP increases as the aqueous humor is no longer able to properly drain from the anterior chamber. In this model, damage in the ONH occurs. Enlarged axons with accumulation of mitochondria as well as membrane-bound vesicles associated with astrocytes are seen in this model (Morrison et al., 1997). This model is cost-effective and highly reproducible. More importantly, this model produces many of the important hallmarks of glaucoma including progressive death of RGCs and axonal degeneration (Almasieh et al., 2010). On the other hand, this model requires a high level of dexterity by the experimenter to construct the glass needles and tubing necessary to deliver the saline solution as well as to perform the microinjection of saline into the vein.

I.2.3.2.2. Laser treatment of limbal tissue

In this model, an argon or diode laser is applied to the TM and/or episcleral and limbal veins (WoldeMussie et al., 2001; Levkovitch-Verbin et al., 2002). This treatment successfully produces elevated IOP in rats by coagulating the limbal vasculature and inducing scarring in the anterior chamber angle resulting in resistance to the aqueous humor outflow (Morrison et al., 2008). This technique produces peaks of IOP between 35-49 mmHg and remains elevated for at least 3 weeks. During this time period a substantial loss of RGCs occurs. This method is also reliable and simple, but requires specialized equipment to be performed. In addition, this is not a chronic model and thus, maintaining the OHT for more than 3 weeks requires further laser treatments, which increases the occurrence of corneal decompensation (corneal edema resulting from failure of the corneal endothelium to maintain its transparency) (Levkovitch-Verbin et al., 2002). Laser photocoagulation has also been performed in mice to decrease aqueous humor outflow and elevate the IOP. This treatment in C57BL/6J mice leads to a marked RGC loss and axonal degeneration (Gross et al., 2003; Yun et al., 2014).

I.2.3.2.3. Episcleral vein cauterization

In this model, elevated IOP is achieved by cauterizing several episcleral veins. IOP is elevated up to six-fold compared with the untouched eye and persist for 6 month after the procedure (Sawada and Neufeld, 1999). Although some controversial data have been reported concerning the OHT variability and the mechanisms underlying the elevation of IOP (Morrison et al., 2008), at the present time, this technique is successfully used by several laboratories (Roh et al., 2012; Bai et al., 2014).

I.2.3.2.4. Occlusion models based on microbead injection

In this model, polystyrene beads are injected into the anterior chamber. The microbeads move to the iridocorneal region where the trabecular meshwork is located, blocking aqueous humor outflow. There is an approximate 30% increase in IOP (Sappington et al., 2010). This model results in RGC death and optic nerve damage including axonal degeneration. Relative to the previous models of glaucoma induction, this model is easy to perform. The major disadvantage of polystyrene beads is the potential for the beads to reflux after the injection. When reflux occurs, the quantity of beads varies per injection creating variability in the results. To avoid the reflux, some groups inject a viscoelastic solution immediately following the injection of microbeads (Cone et al., 2010). Others use magnetic microspheres (Samsel et al., 2011). Another disadvantage of microbead occlusion models is that the injected beads accumulate in the anterior chamber and block the passage of light, therefore *in vivo* retinal imaging or/and behavioral experiments are not possible.

I.2.3.2.5. Genetic model: the DBA/2J mouse

The DBA/2J mice spontaneously develop a chronic age-related glaucoma phenotype. DBA/2J mice have homozygous mutations in two genes: tyrosinase-related protein 1 (*Tyrp1)* and glycosylated transmembrane protein nmb (*Gpnmb*). Mice homozygous for mutations in these two genes exhibit pigment dispersion, iris transillumination, iris atrophy and anterior synechiae (Anderson et al., 2002). Formation of synechiae is the result of the accumulation of dispersed pigment into the anterior chamber, contributing to the blockade of drainage structures and the subsequent elevation of IOP (McKinnon et al., 2009; Johnson and Tomarev, 2010). The main advantage of this model is that age-related IOP occurs spontaneously, which leads to RGC death and axonal degeneration. However, only 70% of mice develop OHT. Thus, a large number of animals is required because of the high degree of phenotypic variability and asymmetry. Also, a long incubation time of 9 to 12 months is required and many of these mice develop corneal abnormalities.

I.3. The neuroinflammatory response in glaucoma

A considerable amount of data supports the presence of an early inflammatory response in the glaucomatous retina in both human patients and in animal models. However, it is still uncertain whether early inflammatory signaling is beneficial or detrimental for RGCs following axonal injury (Nickells et al., 2012). The inflammatory response is mediated by the interaction of neurons with Müller cells, astrocytes, and microglia (Fig. 3). Here, I will discuss the role of glial cells in the neuroinflammatory response in glaucoma.

I.3.1. Reactive gliosis

I.3.1.1. Macroglial response

At the onset of disease, the response of Müller cells has a neuroprotective effect on the retina. Indeed, injury to the retina initially results in physiological and molecular changes in Müller cells that attempt to re-establish homeostasis in the retinal tissue. However, when the insult persists, these changes may become detrimental and even accelerate the neurodegenerative process. This transition is accompanied by dysregulation of the blood-retinal-barrier, expression of proinflammatory factors, and infiltration of immune cells (Bringmann et al., 2009). The over-expression of glial fibrillary acidic protein (GFAP) and phosphorylation of extracellular signal-regulated kinases (ERK) are considered markers of reactive Müller cells which are detected between 2 and 3 days after OHT induction (Kanamori et al., 2005; Bringmann et al., 2006). In fact, GFAP upregulation in both astrocytes and Muller cells is considered an indicator of retinal injury and glial cell activation (Luna et al., 2010). It has been reported that after injury, Nuclear Factor-kappa B (NF-κB) is activated in Müller cells, triggering over-expression of tumor necrosis factor alpha (TNFα) (Lebrun-Julien et al., 2009). Moreover, Müller cells contribute to RGC dysfunction by secreting toxic factors such as alpha2-macroglobulin, and nitric oxide (NO) (Tezel and Wax, 2000; Bai et al., 2011). Müller cells also contribute to the RGC death in glaucoma by dysregulation of the glutamate-glutamine cycle, in which glutamate uptake and glutamine synthetase activity are both reduced (Moreno et al., 2005). Indeed, GLAST knockout mice exhibit spontaneous RGC soma and axon degeneration and are used as a model of NTG (Harada et al., 2007). Recently, it has been reported that swelling and proinflammatory cytokines over-activate the transient receptor potential vanilloid type 4 (TRPV4) channels at the endfoot of Müller cells generating calcium

ions (Ca^{2+}) waves that affect the interglial, gliovascular and neuroglial signals (Ryskamp et al., 2014). Although new insights regarding the role of Müller cell in retinal diseases are increasing, the processes by which these cells establish dialogue with other cells in the retina remain to be clarified.

Without a doubt, reactive astrocytes, which are abundant in the NFL and the ONH, are involved in several mechanisms that cause RGC death and axonal degeneration. Following IOP elevation, astrocytes show morphological changes such as cellular hypertrophy likely due to the mechanical stress induced by the lamina cribrosa (Yu et al., 2013). These morphological changes are accompanied by an upregulation of GFAP, matrix metalloproteinases, and extracellular matrix components such as laminin, tenascin C, and proteoglycans (Hernandez et al., 2008). All of these changes result in ONH remodelling. Furthermore, reactive astrocytes migrate from the cribiform plates to the nerve bundles, where they cause RGC death by releasing NO and TNFα (Liu and Neufeld, 2000, 2004; Tezel et al., 2004). The upregulation of phagocytosis-related Mac-2 mRNA in reactive astrocytes located in the laminar and orbital regions of the optic nerve suggest that astrocytes increase their phagocytic activity in glaucomatous retina (Nguyen et al., 2011). Reactive astrocytes are involved in several mechanisms leading to RGC death and axon degeneration in the NFL as well as ONH, where they are abundant. Interestingly, it has been shown that morphological integrity of ONH astrocytes are preserved by blocking excitotoxicity with memantine. Furthermore, memantine treatment increased mitochondrial fission, volume density and length in astrocytes of glial lamina in glaucomatous mice. This protection is mediated by preventing mitochondria dysfunction and decreasing the formation of autophagosome/autolysosome (Ju et al., 2014).

These findings show mitochondria dysfunction in ONH astrocytes and axons have a detrimental effect on RGC survival.

I.3.1.2. Microglia response

Similar to Müller cells, reactive microglia appear to initially play a protective role against inflammation early after injury. However, the persistence of elevated IOP and/or other contributing factors switch the initial protective response into a detrimental one that promotes inflammation (Nickells et al., 2012). Furthermore, it has also been suggested that both protective and detrimental effects may occur at the same time (Seitz et al., 2013). Optic nerve lesions cause microglial proliferation (Wohl et al., 2010). In addition, the microglia become reactive in both human glaucoma patients (Yuan and Neufeld, 2001) and in experimental animal models of OHT (Bosco et al., 2011). Interestingly, upon neuronal degeneration, activation of microglia is not only found in the retina, but also in the lateral geniculate nucleus (LGN) and correlates with neuronal degeneration (Shimazawa et al., 2012). Activation of microglia involves morphological changes, cell displacement, and increased phagocytic capacity. Microglial phagocytosis is regulated by several signals received form the environment involving the complement components C1q and C3. In glaucoma, C1q is upregulated in the synapses, axons and cells bodies before synapse loss and RGC death. These findings suggest that microglia phagocytosis occurs in neurons previously marked by complement (Stevens et al., 2007). Also, microglial activation results in upregulation of several proteins including those that promote inflammation including TNFα and IL-6 (Yuan and Neufeld, 2000; Sappington and Calkins, 2006; Sappington et al., 2006; Chi et al., 2014), complement proteins (Stephan et al., 2012) and other neurotoxic factors (Colton and Gilbert, 1987; Neufeld et al., 1997; Neufeld et al., 2002; Vidal et al., 2006). When microglia become

activated, their cell body size increases and they appear round as the ramification is lost (Ransohoff and Perry, 2009). Interestingly, a higher number of reactive non-ramified microglia has been observed around the ONH in several models of OHT (Taylor et al., 2011; Roh et al., 2012), suggesting that after injury, microglia migrate to the ONH. In other animal models of glaucoma, reactive microglia have been detected in the contralateral non-treated retina (Gallego et al., 2012; Rojas et al., 2014). Inhibiting the activation of microglia using minocycline leads to RGC protection after optic nerve lesion (Levkovitch-Verbin et al., 2006; Bosco et al., 2008), suggesting that activation of microglia is involved in RGC loss after optic nerve injury. Moreover, blocking microglia activation by deleting the receptor CD11b results in neuroprotection in an experimental glaucoma model (Nakazawa et al., 2006).

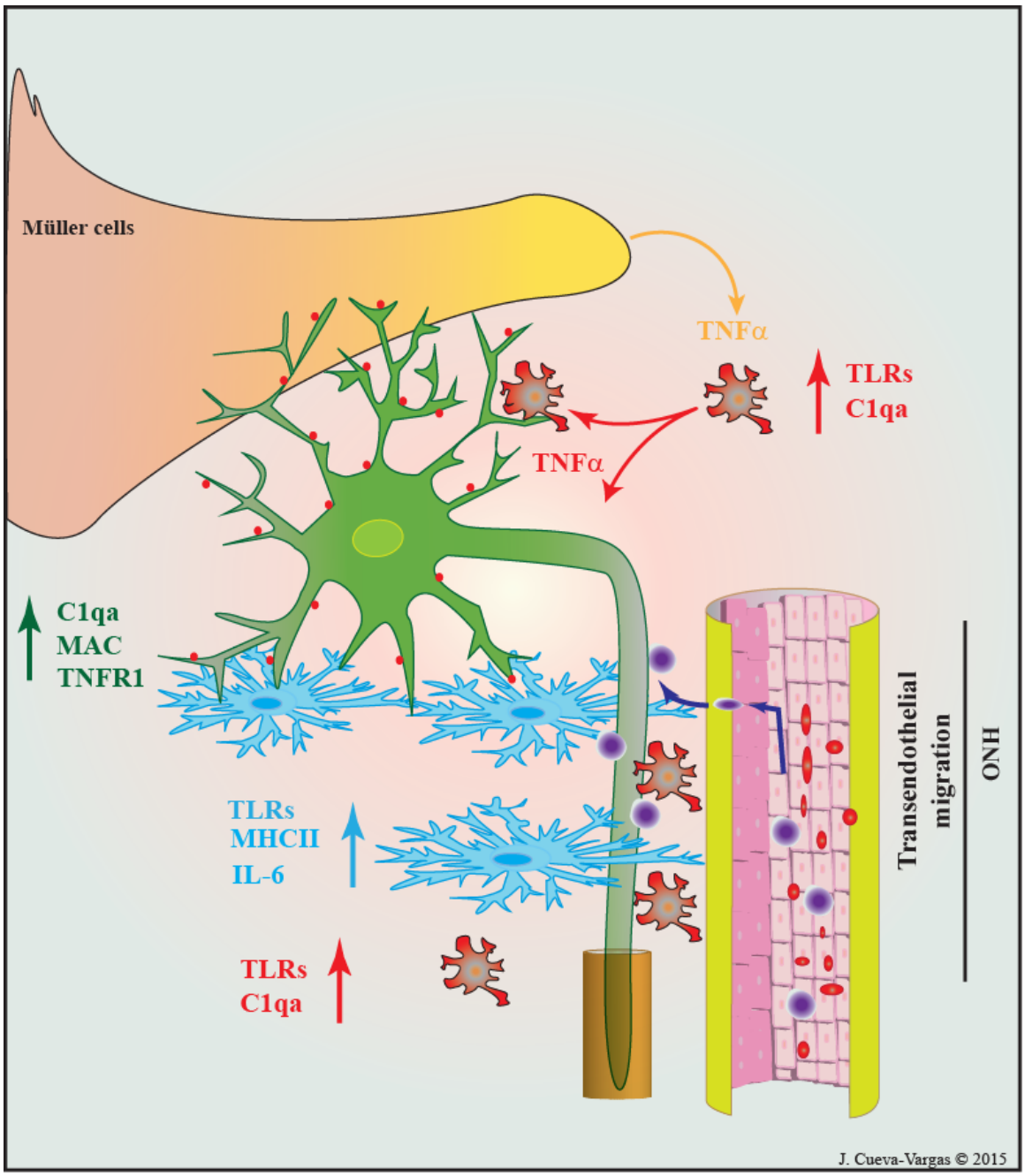

Müller cells
TNFα
TLRs
C1qa
TNFα
C1qa
MAC
TNFR1
TLRs
MHCII
IL-6
TLRs
C1qa
Transendothelial migration
ONH
J. Cueva-Vargas © 2015

Figure 3. Model of early neuroinflammatory responses in glaucoma mediated by resident glial cells. Early after OHT induction TNFα is secreted by Müller cells. TNFα has detrimental effects on RGCs (green) and also could activates microglia (red) and astrocytes (light blue) through TNFR1**.** Glial cells express pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). After OHT induction, TLRs activate the production and secretion of new pro-inflammatory cytokines, increasing the neuroinflammatory response. Cytokines and chemokines induce changes in the endothelial cells and the retinal-blood-barrier integrity, allowing the infiltration of blood-derived immune cells (purple), amplifying the immune response in the retina. Elevated IOP also leads to upregulation of C1qa in microglia, but deposition of C1qa was also observed in RGC dendrites. Source: Jorge Luis Cueva Vargas, modified from (Soto and Howell, 2014). Cold Spring Harbor Perspectives in Medicine.

I.3.2. Proinflammatory cytokines

Cytokines are comprised of a family of soluble proteins that are released by immune cells. In the CNS, neurons, glia, and endothelial cells produce and release cytokines, which act in a paracrine or autocrine manner. Cytokines modulate neuronal activity by promoting the release of neuroactive molecules such as NO, glutamate, and neurotrophins. In addition, cytokines bind to their cognate receptors in the target cells to further activate various signalling pathways (Vezzani and Viviani, 2014). Here, I will discuss the pro-inflammatory cytokines that are the most relevant to glaucomatous neurodegeneration,, including TNFα, interleukin-1β (IL-1β), interleukin-6 (IL-6) and macrophage migration inhibitory factor (MIF).

I.3.2.1. Tumor necrosis factor alpha

TNFα, a homotrimer 17 KDa protein, is first synthesized as a trans-membrane peptide which is then cleaved by the matrix metalloprotease TNFα-converting enzyme (TACE) to generate the soluble form of TNFα (Moss et al., 1997). Both trans-membrane TNFα and soluble TNFα are physiologically active and play different roles *in vivo*, depending on their binding to TNFα receptor 1 (TNFR1) or TNFα receptor 2 (TNFR2). Soluble TNFα has higher affinity for TNFR1 while trans-membrane TNFα has higher affinity for TNFR2 (Tartaglia and Goeddel, 1992; Idriss and Naismith, 2000). The two receptors have different downstream-signal transduction effects.

TNFR1 is associated with cytotoxic and apoptotic effects conferred by the cytoplasmic death domain. Briefly, TNFα binding induces TNFR1 trimerization and recruitment of TNF receptor-associated death domain (TRADD) followed by additional recruitment including, receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2) and Fas-

associated death domain (FADD). This complex leads to the recruitment and activation of caspase-8 and 10, which in turn promotes the initiation of apoptosis (Chen and Goeddel, 2002; Micheau and Tschopp, 2003). TNFR1 signaling can also mediate survival by activation of NF-κB. Indeed, TRADD-TRAF2/5 complex is able to recruit and ubiquitinate RIP in the presence of the cellular inhibitor of apoptosis 1/2 (c-IAP 1/2). RIP can also be ubiquitinated in the absence of TRADD. Ubiquitinated RIP can phosphorylate the catalytic IκB kinase (IKK), which in turn phosphorylates the inhibitor of kappaB-alpha (IκBα), leading to activation of NF-κB (Naude et al., 2011). TNFR2 does not possess death domain and trans-membrane TNFα/TNFR2 binding leads to anti-apoptotic signals and NF-κB activation through TRAF2 recruitment (Fontaine et al., 2002; Olmos and Llado, 2014). However, the role of TNFR2 in cell death has already been documented (Depuydt et al., 2005; Nakazawa et al., 2006). In 2006, the group of Benowitz and colleagues reported that elevated IOP does not lead to RGC death in TNR2-deficient mice compared to TNFR1-deficient mice. They argue that TNFα exerts its detrimental effect via TNFR2 expressed on microglia which release reactive oxygen species and TNFα (Nakazawa et al., 2006). To better understand how TNFR2 is involved in cell toxicity further research is still needed.

In the CNS, TNFα is endogenously expressed at low levels and plays an important role in synaptic plasticity (Stellwagen, 2011), glial transmission (Beattie et al., 2002), learning and memory (Baune et al., 2008) and sleep (Krueger, 2008). Under pathological conditions, elevated levels of TNFα has detrimental effects in many neurodegenerative conditions including Alzheimer's disease (McAlpine et al., 2009), Parkinson's disease (McCoy et al., 2006), multiple sclerosis (Eugster et al., 1999), ischemic and traumatic injuries (Esposito and Cuzzocrea, 2009), chronic pain (Hess et al., 2011), and epilepsy (Vezzani et al., 2008).

Despite these toxic effects of TNF-α, the protective ability of glial-derived TNFα has also been reported (Lambertsen et al., 2009). These seemingly contradictory effects may be dependent on the compensatory effects produced by other cytokines such as IL-1β, the differential expression of TNFR1 and TNFR2, and by the variability, time course, and magnitude of the pathology.

In the retina, the physiological role of endogenous TNFα is poorly understood. Intravitreal injection of TNFα has been shown to be toxic for RGCs and their axons in the optic nerve (Nakazawa et al., 2006; Lebrun-Julien et al., 2010). Indeed, high levels of TNFα are observed in experimental (Roh et al., 2012) and human glaucoma (Yuan and Neufeld, 2000). In addition, high levels of TNFα have been detected in the aqueous humor of glaucoma patients (Balaiya et al., 2011). Furthermore, inhibiting TNFα by using etanercept, an inhibitor of soluble and transmembrane TNFα, promotes protection of RGC soma and axons in experimental glaucoma (Roh et al., 2012). The mechanism by which TNFα promotes RGC loss remains unclear. One potential mechanism suggests that TNFα mediates the insertion of Ca^{2+}-permeable AMPA receptors into the membrane during excitotoxic injury, leading to RGC death (Lebrun-Julien et al., 2009).

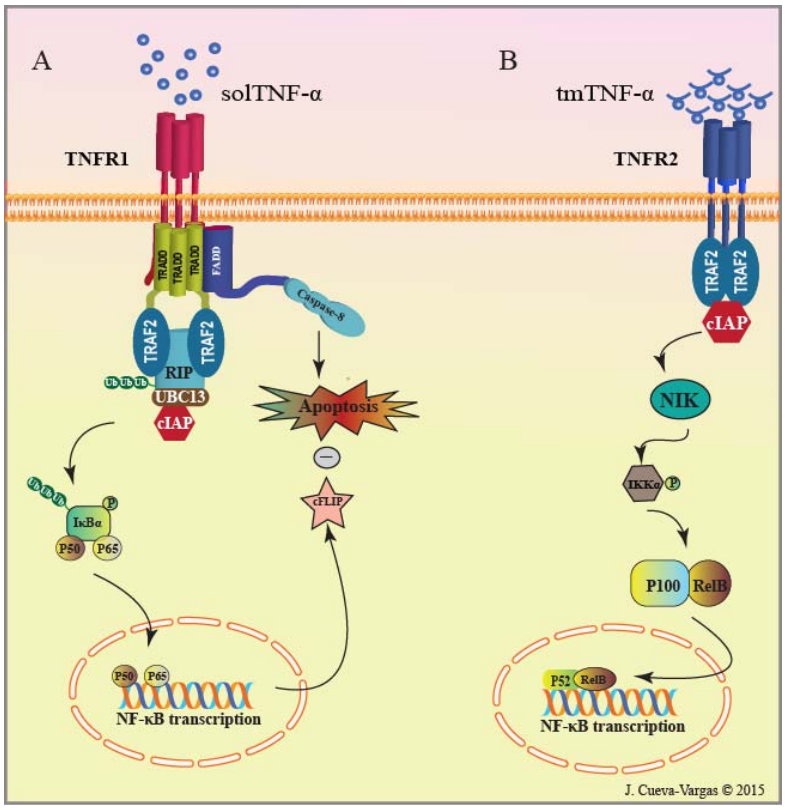


Figure 4. TNFα signal transduction via TNFR1 and TNFR2. A. Binding of soluble TNFα to TNFR1 results in TRADD, RIP and TRAF2 recruitment; subsequently RIP become ubiquitinated and dissociated from TNFR1. The DD of TRADD binds FADD, resulting in caspase-8 recruitment and apoptosis. On the other hand, polyubiquitinated RIP1 leads to phosphorylation of IκBα, allowing its degradation via the ubiquitin-proteasome pathway. Thus, NF-κB translocates to the nucleus and initiates transcription. NF-κB activation blocks apoptosis by producing cFLIP leading to cell survival. B. Binding of trans-membraneTNFα to TNFR2 leads to the recruitment of TRAF2 and cIAP1/2 and the subsequent phosphorylation of IKKα and the NF-κB precursor protein p100. p100 is then cleaved, resulting in activation of p52-containing NF-κB. Source: Jorge Luis Cueva Vargas.

I.3.2.2. Interleukin-1β

IL-1β belongs to the IL-1 family which is involved in inflammation and host defense. The precursor factor (pro-IL-1β) is cleaved by caspase-1 to produce the active form of the protein which is 17 KDa (Thornberry et al., 1992). IL-1β exerts its biological function by binding to IL-1R. This interleukin is constitutively expressed in the CNS and responds to stimulus coming from the neuroendocrine system and the local tissue environment. Thus, IL-1β participates in a variety of processes including the regulation of appetite, body temperature, epilepsy, sleep/wake cycle, and the modulation of inflammatory processes in the event of injury or disease (Shaftel et al., 2008).

High levels of IL-1β have been reported in the cerebrospinal fluid of patients with brain injury and stroke, Alzheimer's disease, Parkinson disease, multiple sclerosis, and epilepsy. Inhibiting IL-1β action using an antagonist (IL-1ra) protects against neuronal injury (Allan et al., 2005). Although the exact mechanisms by which IL-1β modulates toxicity remain to be elucidated, a functional interaction between IL-1β/IL-1R complex to N-methyl-D-aspartate receptors (NMDA) receptors appears to be involved in the promotion of cell death. In fact, IL-1R interacts specifically and physically with GluN2B subunit of the NMDA receptor. Furthermore, high levels of IL-1β have been proposed to enhance NMDA-mediated neuronal Ca^{2+} influx leading to excitoxicity (Vezzani and Viviani, 2014).

In the retina, IL-1β expression increases after retinal/ischemia reperfusion. This damage is reduced by inhibiting IL-1β activity further confirming that high levels of IL-1β have toxic effects on retinal neurons (Yoneda et al., 2001; Chi et al., 2014). Recently, it has been shown that IL-1β protein is increased in microglia in response to excitotoxicity and IL-1β

mRNA is upregulated when IOP increases (Chidlow et al., 2012). Furthermore, high levels of IL-1β have been reported in the aqueous humor of patients with POAG (Takai et al., 2012). In light of these reports, the role of IL-1β after OHT induction requires further investigation.

I.3.2.3.Interleukine-6

In the CNS, astrocytes and microglia are the major source of IL-6; however, neurons can also synthesize IL-6 paricularly in conditions of stress as injury or sustained neuronal activity. IL-6, a 21-28 KDa protein, utilizes the signal transducing receptor subunit gp130. The IL-6-IL-6R complex binds to two molecules of gp130, also known as CD130, to activate signal transduction pathways including janus kinase/signal transducer and activator of transcription (JAK/STAT), ERK and phosphatidylinositide 3-kinases (PI3K) (Scheller et al., 2011). IL-6 has a wide-range of roles in neuronal physiology, neurodevelopment, neuroprotection, and neurotoxicity (Juttler et al., 2002; Gruol, 2014) Neurotoxic effects of IL-6 have been observed in Alzheimer's disease, multiple sclerosis, Parkinson's disease, Huntington's disease, stroke as well as some psychiatric disorders, such as depression (Erta et al., 2012). In contrast, some reports have demostrated that IL-6 mediates neuronal protection (Sanchez et al., 2003; Sappington et al., 2006; Liu et al., 2011; Perigolo-Vicente et al., 2013) and axon regeneration (Leibinger et al., 2013).

In the retina, IL-6 expression is observed in microglia during ischemia-reperfusion injury (Sanchez et al., 2003). However, in experimental glaucoma, IL-6 is mainly expressed by RGCs and it is anterogradely transported to the optic nerve (Chidlow et al., 2012). In addition, high levels of IL-6 mRNA are found in the ONH early after glaucoma induction

(Johnson et al., 2011). Although the mechanism of action is not well defined, these results suggest that IL-6 is dynamically involved during the development of the disease.

I.3.2.4.Macrophage migration inhibitory factor

MIF was first described as a T-cell-secreted cytokine that inhibited the random migration of macrophages from capillary tubes (David, 1966). MIF is a 12.5 KDa protein that is highly conserved. MIF is highly conserved and it is constitutively expressed in many cells types and tissues. Macrophages are the major source of MIF (Calandra et al., 1994). Currently, three receptors that interact with MIF have been identified, CD74, CXCR2 and CXCR4 (Leng et al., 2003; Bernhagen et al., 2007). CD74 is the MHC class II invariant chain. MIF-CD74 binding results in activation of the ERK 1/2, mitogen-activated protein kinases (MAPK), and activator protein 1 (AP-1) pathways (Leng et al., 2003). MIF binding to CXCR2 and CXCR4 results in trafficking of immune cells to the inflammation site (Glabinski et al., 2000; Bernhagen et al., 2007; Carlson et al., 2008).

In the brain, MIF expression increases after exposure to inflammatory stimuli (Bacher et al., 1998; Ogata et al., 1998; Bacher et al., 2002). In response to inflammatory factors such as TNFα, MIF is released to mediate different cellular events including cell survival, phagocytosis, proinflammatory cytokine production, and inducible nitric oxide synthase (iNOS) (Onodera et al., 1997; Mitchell et al., 2002). MIF has been involved in adult neurogenesis, learning and memory, depression (Conboy et al., 2011) and stroke (Inacio et al., 2011), and can participate in the pathogenesis of Alzheimer's disease and EAE (Popp et al., 2009; Bacher et al., 2010; Cox et al., 2013).

In the retina, MIF is expressed by astrocytes and Müller cells (Matsuda et al., 1997). However, the function of MIF in the retina is largely unknown. Recently, in a diabetic retinopathy model, its receptor CD74 was has been detected in activated microglia (Wang et al., 2014b), suggesting that MIF might play a role in the retina.

I.3.3. Role of the complement cascade

In the immune system, complement proteins aid phagocytic cells clear up cell debris and pathogens. The complement system is comprised of three primary pathways: the classical pathway, the lectin pathway, and the alternative pathway. All three pathways generate C3 and C5 convertases, as well as bound C5b, which is converted into the membrane-attack complex (MAC). The classical pathway, which first component is C1q, has been implicated in glaucoma and synaptic pruning (Stevens et al., 2007). Indeed, C1q is upregulated in animal glaucoma models and human glaucoma (Stasi et al., 2006). In the glaucomatous DBA/2J mice, where synapse loss precedes neuronal death, high levels of Cq1 and C3 are found in the IPL at the early glaucoma stages. Moreover, neuronal protection is observed in DBA/2J mice deficient in C1q (Howell et al., 2011). In the IPL, binding of C1q and C3 at synapses leads to activation of the complement cascade. Subsequently, activated microglia may phagocytize C1q and/or C3-tagged synapses.

Independently of microglia activation, loss of synapses may be triggered by MAC formation (Rosen and Stevens, 2010). Importantly, MAC formation was increased in glaucomatous RGCs in human retinas and in experimental models of OHT (Kuehn et al., 2006; Jha et al., 2011; Soto and Howell, 2014). In addition, MAC formation increases Ca^{2+} influx in RGCs, leading to neuronal apoptosis. On the other hand, depletion of the

complement system blocks Ca^{2+} influx in RGCs (Jha et al., 2011), confirming the pathological role of complement activation in glaucoma. Complement activation has also been reported in other neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Stephan et al., 2012).

I.3.4. Role of the phosphodiesterase 4 in neuroinflammation

The phosphodiesterases (PDEs) superfamily comprises 11 gene families (PDE1-PDE11), which are encoded by more than 20 genes. Each family represents 1 to 4 genes, resulting in more than 50 functionally different PDE proteins (Lugnier, 2006). These families are grouped according to substrate specificity, sensitivity to inhibitors, regulations and amino acid sequences. PDEs regulate the levels of 3'-5'-cyclic adenosine monophosphate (cAMP) and/or 3'-5'-cyclic guanosine monophosphate (cGMP) by hydrolyzing them. In the CNS, PDE4 is the most abundant and it is characterized by being only cAMP specific (Bolger et al., 1994). PDE4 family is encoded by four different proteins: PDE4A, PDE4B, PDE4C and PDE4D. Each gene contains alternative mRNA splicing, resulting in more than 30 isoforms (Lugnier, 2006). PDE4C is not expressed in the CNS (Lamontagne et al., 2001; Whitaker and Cooper, 2009).

PDE4 is the most studied PDE and its presence has been reported in the retina (Whitaker and Cooper, 2009). PDE4 is involved in a number of pathological processes including neuroinflammation through modulation of cAMP (Fig. 5). An increase of cAMP levels through PDE4 inhibition results in activation of the cAMP-dependent protein kinase A (PKA), exchange proteins directly activated by cAMP (EPAC1 and EPAC2) or cAMP-gated channels, which are implicated in a wide range of cellular functions. PKA is the most

important target of cAMP signaling. Upon binding of cAMP, inactive PKA becomes active, which in turn is able to phosphorylate serine and threonine residues on the transcription factor cAMP-response element binding protein (CREB) (Gerlo et al., 2011). Phosphorylated CREB can then regulate several molecules including BDNF, which enhances neuronal survival (Di Polo et al., 1998; Lipsky and Marini, 2007) (Fig. 5A).

cAMP has also been reported to have an anti-inflammatory effect by interfering with the proinflammatory transcription factor NF-κB mainly by blocking IKKβ activity and increasing cellular levels of IκB (Fig. 5B) (Gerlo et al., 2011). In addition, activated PKA has also been shown to be anti-inflammatory most likely by attenuating activation of NF-κB p65 (Parry and Mackman, 1997), which is involved in the upregulation of proinflammatory cytokines such as TNFα in Müller glia (Lebrun-Julien et al., 2009). Of interest, increasing the level of cAMP using PDE4 inhibitors has been shown to attenuate the inflammatory response in a number of neurodegenerative diseases including Alzheimer's disease (Wang et al., 2012), spinal cord injury (Schaal et al., 2012), and multiple sclerosis (Rolan et al., 2009). Furthermore, PDE4 inhibitors also reduces glial activation (Kiebala and Maggirwar, 2011; Ghosh et al., 2012), the main source of proinflammatory cytokines in the nervous system (Soto and Howell, 2014).

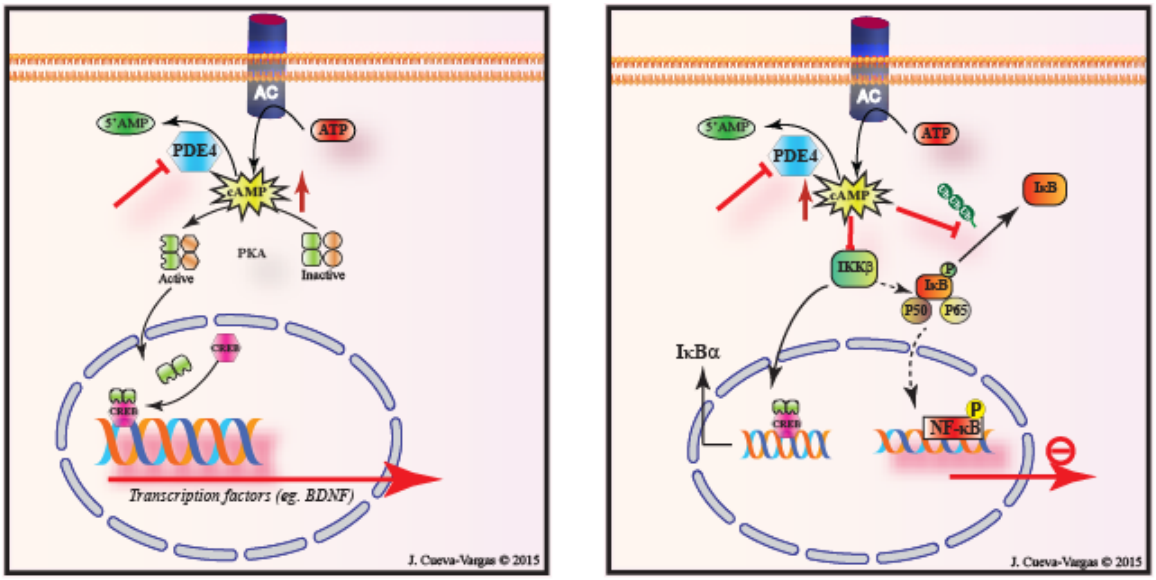


Figure 5. Phosphodiesterase type-4-mediated signal transduction processes. A. PDE4 degrades cAMP to 5'AMP. An inhibition of PDE4 triggers higher level of cAMP, which can bind to PKA holoenzyme releasing the two catalytic subunits. The activated subunits then translocate to the nucleus where they can phosphorylates multiple substrates among them the transcription factor CREB. Phosphorylated CREB induces the transcription of a number of target genes including BDNF. B. Elevated intracellular cAMP inhibits NF-κB activity by increasing cytoplasmic levels of IκKα, blocking IKKβ activity and increasing IκB levels by blocking ubiquitinylation of IκB. Source: Jorge Luis Cueva Vargas.

I.4. Excitotoxic damage in glaucoma

Glutamate is the primary excitatory neurotransmitter in the CNS and, under physiological conditions, it is a key regulator of neuronal transmission (Thoreson and Witkovsky, 1999). Dysregulation of glutamate signaling has been associated with a variety of disorders including ischemia induced by retinal or choroidal vessel occlusion, diabetes retinopathy and glaucoma (Casson, 2006; Ishikawa, 2013). Glutamate is released by presynaptic terminals and binds to glutamate receptors on the dendrites of postsynaptic neurons leading to membrane depolarization. Levels of glutamate are tightly regulated as high levels are toxic for neurons (Ishikawa, 2013). In fact, an injection of glutamate or its analog NMDA into the eye has been reported to induce death of retinal neurons (Lucas and Newhouse, 1957). Moreover, the use of glutamate receptor antagonists has been shown to revert this toxicity. Collectivelly, these findings suggest that glutamate mediates toxicity by a similar mechanism found in excitatory glutamatergic synapse (Olney, 1969). Indeed, John Olney has demonstrated that the postsynaptic neurons were the targets of this cell death, where the dendrites of target neurons were swollen while the pre-synaptic endings were spared (Olney, 1969). In glaucoma, glutamate-induced excitotoxicity has been proposed to mediate Ca^{2+}-dependent apoptosis of RGCs (Fig. 6). Indeed, excitotoxicity occurs when there is prolonged or excessive activation of glutamate receptors resulting in excessive accumulation of intracellular Ca^{2+} and subsequent activation of Ca^{2+}-dependent apoptotic processes (Qu et al., 2010). Three mechanisms have been proposed to mediate excitotoxicity (Ishikawa, 2013). First, excitotoxicity may occur when there is impairment of glutamate transport. Downregulation of GLAST expression has been proposed to mediate neuronal loss in experimental glaucoma and in human glaucoma patients (Naskar et al., 2000; Martin et al.,

2002). Interestingly, GLAST-deficient mice show spontaneous RGC death and axonal degeneration without elevated IOP (Harada et al., 2007). In addition, GLT-1, a glutamate transporter located in cones photoreceptors and bipolar cells, appears to contribute to RGC death (Martin et al., 2002). Second, an impairment of the glutamate/glutamine cycle leading to excitotoxicity has been reported in experimental glaucoma (Moreno et al., 2005). Third, reduction of intracellular ATP mediated by mitochondrial impairment also affects neuron survival and axonal transport through glutamate excitotoxicity. A decrease in ATP levels results in dysfunction of the Na^+/K^+ exchanger, resulting in an increase in intracellular Na^+ and a subsequent release of glutamate (Rossi et al., 2000).

I.4.1. Glutamate receptors

To mediate excitatory neurotransmission, glutamate acts on different membrane receptors that include ionotropic and metabotropic glutamate receptors (mGluR). Ionotropic receptors act postsynaptically where they mediate fast synaptic transmission. Ionotropic receptors can also mediate neurotransmitter release at the presynaptic site (Pinheiro and Mulle, 2008). mGluRs function as a single protein and exert their effect by influencing several intracellular second messenger systems via interaction with membrane-bound G proteins. Ionotropic glutamate receptors include: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors.

I.4.1.1. NMDA receptors

Functional NMDA receptors are heterotetramers. NMDA receptors exhibit unique properties depending on the type and combination of subunits present in the heterotetramer receptor complex There are fourteen different subunits including: eight GluN1 subunits, four

GluN2 subunits (GluN2A-D), and two GluN3 subunits (GluN3A-B), which are encoded by one, four, or two genes, respectively (Paoletti et al., 2013; Glasgow et al., 2014). The four GluN2 subunits are considered to play a particularly important role in determining receptor function.

At resting membrane potential, NMDA receptors are blocked by magnesium ions (Mg^{2+}). Activation of NMDA receptors requires glutamate and either glycine or D-serine as co-agonists. Upon activation of the NMDA receptor, the Mg^{2+} block is expelled, the ion channel opens and there is an influx of Na^{+} and Ca^{2+} and an efflux of K^{+}, causing a transient depolarization and activation of Ca^{2+}-dependent signaling pathway. This event is crucial for neuronal survival and plasticity. The specific downstream effect is determined by alternative splicing and subunit composition of the NMDA receptor (Zito, 2009).

The pathological activation of the NMDA receptors is involved in several diseases of the CNS including cerebral ischemia, traumatic brain injury, Alzheimer's disease, Parkinson's disease, Huntington's disease, depression, pain, schizophrenia (Paoletti et al., 2013). In the retina, NMDA receptor-mediated excitotoxicity contributes to RGC injury in several diseases including retinal ischemia and glaucoma. Intravitreal injection of NMDA is highly toxic for RGCs partly due to the activation of NF-κB leading to the production of TNFα (Lebrun-Julien et al., 2009). In addition, it has been shown that NMDA toxicity is mediated by the GluN2B subunit of NMDA receptor. GluN2B also mediates RGC loss in $GLAST^{-/-}$ mice, a model of normal tension glaucoma (Bai et al., 2013).

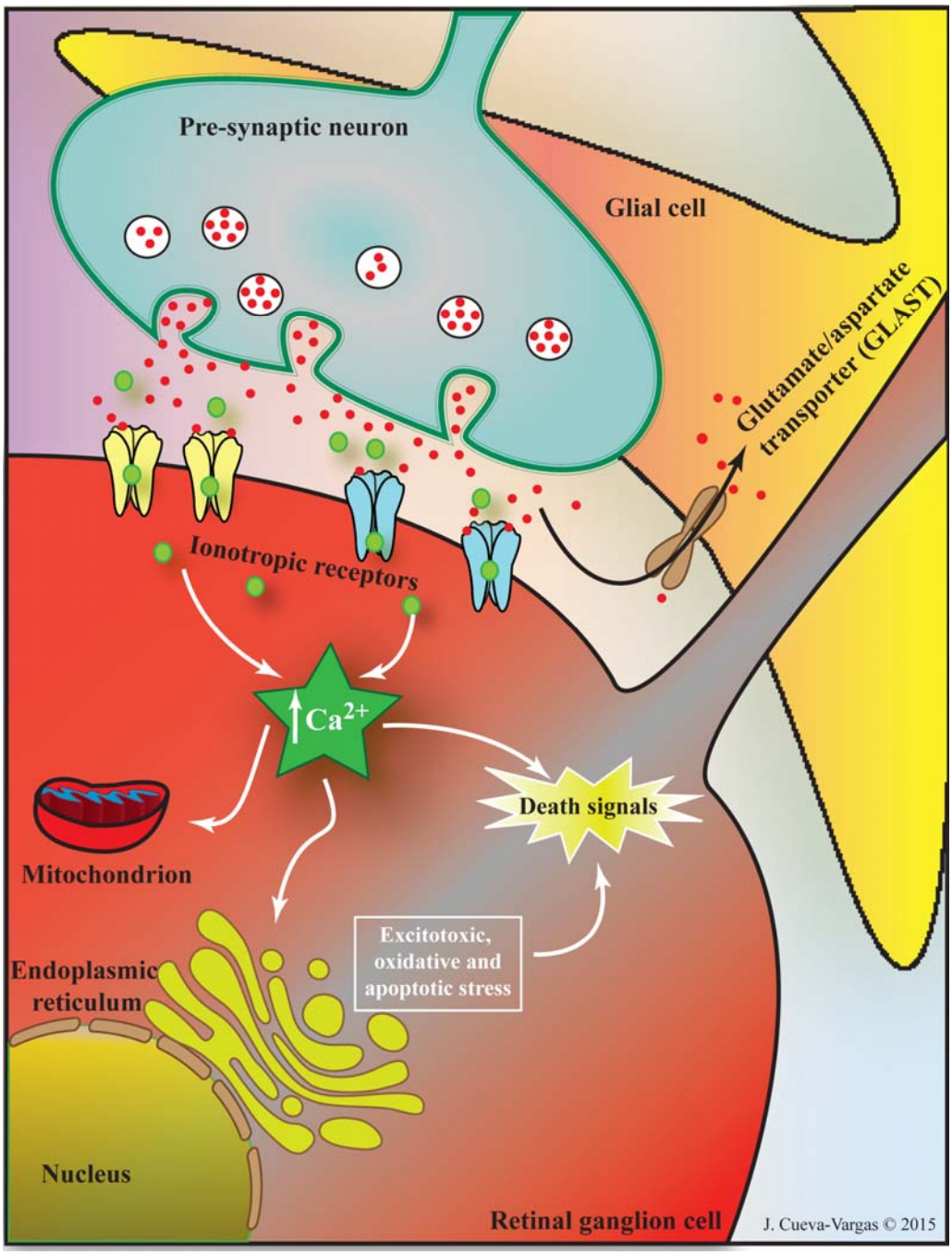

Pre-synaptic neuron
Glial cell
Glutamate/aspartate transporter (GLAST)
Ionotropic receptors
Ca2+
Death signals
Mitochondrion
Excitotoxic, oxidative and apoptotic stress
Endoplasmic reticulum
Nucleus
Retinal ganglion cell
J. Cueva-Vargas © 2015

Figure 6. Classical model of excitotoxicity in the retina. Higher levels of glutamate (red circles) bind to ionotropic glutamate receptors (NMDA and AMPA receptors) allowing an excess Ca^{2+} influx (green circles). Excessive Ca^{2+} entry into neurons stimulates death signals. Ca^{2+} can also be sequestered intracellularly by the mitochondria and endoplasmic reticulum leading to oxidative stress and subsequent activation of apoptotic signals. Source: Jorge Luis Cueva Vargas, modified from (Almasieh et al., 2012).

I.4.1.2. AMPA receptors

Similar to NMDA receptors, AMPA receptors are homomeric or heteromeric proteins made up of four GluA subunits (GluA1-4) (Mellor, 2010). Each subunit has four membrane domains of which three are transmembrane (M1, M3 and M4). M2 is a re-entrant loop and does not traverse the membrane. The glutamate binding site is in the intracellular domain formed by M1, M3 and M4. These glutamine binding sites are known as the S1 and S2 sites. The M2 domain includes the ion selectivity filter. (Fig.7). Upon glutamate binding, the channel opens to allow the influx of Na^+ and the efflux of K^+, mediating fast excitatory synaptic neurotransmission. Under physiological conditions, most AMPA receptors are not permeable to Ca^{2+} due to the presence of the GluA2 subunits. The GluA2 subunit plays an important role in modulating the biophysical properties of the channel's ion permeability. AMPA receptors that do not have the GluA2 subunit are largely considered Ca^{2+}-permeable AMPA receptors (CP-AMPAR). Although CP-AMPAR in the healthy CNS are not permeable to Ca^{2+}, the expression of CP-AMPAR has been reported in several brain regions including inhibitory interneurons and non-neuronal cells such as the Bergmann glial cells (Burnashev et al., 1992). Neuronal CP-AMPARs are found in several aspiny dendrites in the hippocampus, neocortex, striatum, amygdale, spine cord, basal ganglia, brainstem, auditory system and the retina. In these cells, CP-AMPARs have been proposed to mediate Ca^{2+} based plasticity, ensuring precise Ca^{2+} localization (Goldberg et al., 2003). Dysregulation of CP-AMPARs has been proposed to be an underlying factor in the development of several brain diseases including Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis (ALS) (Mellor, 2010).

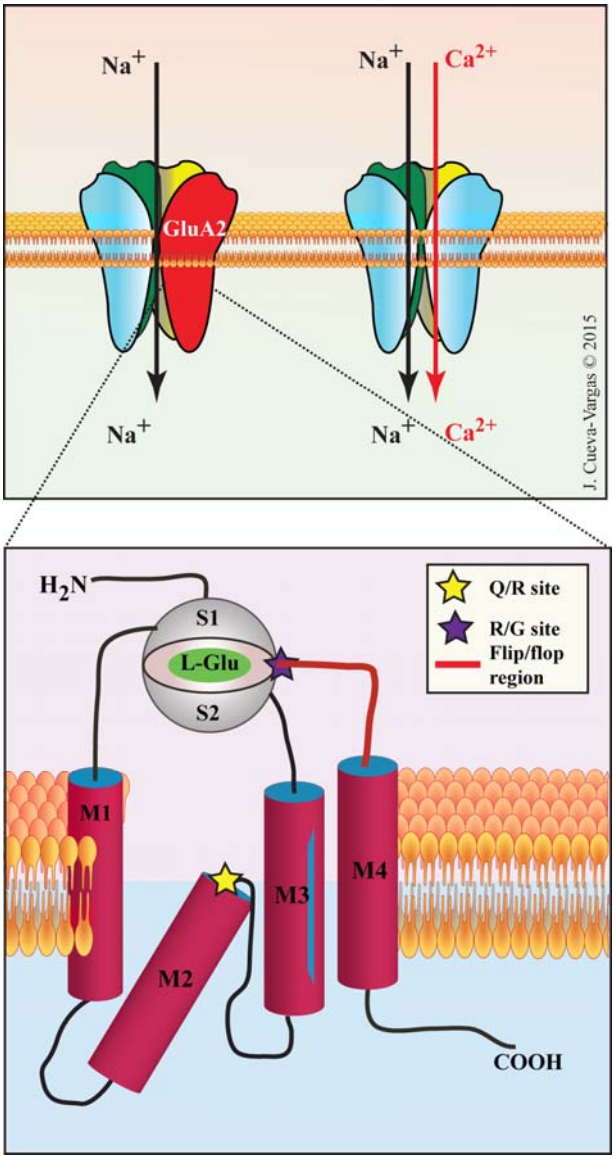


Figure 7. Schematic model of AMPA receptors ionic permeability. A. GluA2-containing AMPA receptor (left) is not permeable to Ca^{2+} while the GluA2-lacking AMPA receptor (right) is highly permeable to both Ca^{2+} and Zn^{2+}. B. Model of GluA2 subunit of AMPA receptor showing the transmembrane domain (M1, M3 and M4) and the re-entrant loop domain (M2). The glutamate binding site is formed by S1 and S2. Two post-transcriptional modifications occur through RNA editing at the Q/R and R/G sites. Source: Jorge Luis Cueva Vargas. Modified from (Mellor, 2010). Copiright © 2010 Future Science Ltd.

I.4.1.2.1. GluA2 subunit modification by RNA editing

As mentioned above, the presence or absence of the GluA2 subunit has important functional consequences for AMPA receptors. AMPA receptors containing the GluA2 subunit are impermeable to divalent cations (Ca^{2+} and Zn^{2+}). Furthermore, they cannot be blocked by intracellular polyamines, have a small unitary conductance, and exhibit a linear current-voltage relationship. All these characteristics are due to the presence of a positively charged arginine residue (R^{+}) in the pore loop of M2 (Fig. 7), specifically at the amino acid position 607. Interestingly, at the DNA nucleotide level, this site is encoded by the glutamine residue (Q). Post-transcriptional RNA editing results in the switch from a glutamine residue to an arginine residue at this site, hence its name Q/R site. RNA editing at the Q/R site occurs when the adenosine deaminase enzyme ADAR2 converts adenosine to inosine in double-stranded RNA substrates (Higuchi et al., 1993; Nishikura, 2010). Briefly, the adenosine (A) present in the CAG codon (encoding glutamine) is converted into inosine (I), resulting in the CIG codon (encoding arginine). This post-transcriptional modification, which occurs exclusively in GluA2, is very efficient as almost 100% of GluA2 mRNA is edited. Deficient RNA editing has been reported in motor neurons in ALS and in CA1 pyramidal neurons after ischemia (Kwak and Kawahara, 2005; Peng et al., 2006) Nonetheless, some studies have reported that GluA2 editing is not altered in a number of diseases (Rump et al., 1996; Kortenbruck et al., 2001).

I.4.1.2.2. Trafficking of GluA2 subunit

Unedited GluA2 enables AMPA receptors to become permeable to Ca^{2+}. Another mechanism involves GluA2 subunit modulation. For example, under physiological conditions,

AMPA receptors are very dynamic and the levels of CP-AMPARs are regulated by a process involving GluA2 trafficking. Recently, it has been reported that NMDA activation of ON RGCs leads to endocytosis of GluA2-containing AMPA receptors and their replacement by CP-AMPARs (Jones et al., 2012). Although this study does not identify a precise mechanism by which endocytosis of GluA2-containing AMPA receptor leads to excitotoxicity, it has been suggested that AMPA receptors are replaced by CP-AMPARs. Under pathological ischemic conditions, the number of CP-AMPARs in hippocampal pyramidal neurons markedly increases (Pellegrini-Giampietro et al., 1997). These data suggest that endocytosis of GluA2 mediates synaptic changes and, in some cases, neuronal toxicity. There are many proteins involved in GluA2 trafficking including the protein interacting with C-kinase-1 (PICK1), which has been associated with GluA2 endocytosis after neuronal trauma. Upon glutamate binding, protein kinase C alpha (PKCα) is activated and trafficked to the plasma membrane by PICK1. This PICK1-PKCα interaction leads to the phosphorylation of GluA2 at serine residue 880. Phosphorylated GluA2 then binds PICK1, resulting in the endocytosis of GluA2 (Bell et al., 2009). Of interest, PICK1-deficient mice show high levels of GluA2 at the surface, suggesting that PICK1 is involved in the internalization and retention of GluA2-containing AMPA receptors within intracellular stores (Clem et al., 2010).

I.4.1.2.3. GluA2-lacking AMPA receptors as mediator of neurotoxicity

In both unedited GluA2 and GluA2-lacking AMPA receptors, toxicity is mediated by the excessive influx of Ca^{2+} and Zn^{2+}. Levels of intracellular $Ca^{2+}$are controlled by neurons through a complex balance of Ca^{2+} influx, efflux, buffering, and storage (Arundine and Tymianski, 2003). During excitotoxicity, high levels of intracellular Ca^{2+} can compromise the integrity of organelles such as the mitochondria and the endoplasmic reticulum. Also, high

levels of intracellular Ca^{2+} can lead to the activation of different proteins including calpains, phospholipase A2, caspases, endonucleases and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Orrenius et al., 2003). The activation of these proteins is often toxic and is a major cause of cell death (Szydlowska and Tymianski, 2010). In experimental glaucoma, calpain activation cleaves the protein phosphatase calcineurin leading to RGC death (Huang et al., 2010). Recently, it has been reported that activation of CP-AMPAR leads to NO production, which activates Src tyrosine kinase resulting in retinal neuron death (Socodato et al., 2012). Toxicity of CP-AMPAR has also been reported in Purkinje neurons (Bliss et al., 2011) and hippocampal pyramidal neurons (Ogoshi et al., 2005). High permeability of CP-AMPARs to Zn^{2+} also appears to contribute to neurotoxicity. In fact, Zn^{2+} translocation and accumulation has been proposed to contribute to mitochondria-mediated neuronal injury. Its effect has been proposed to be more potent than that of Ca^{2+} (Sensi et al., 2000; Weiss and Sensi, 2000)

I.4.1.3. Kainate receptors

Kainate (KA) receptors are tetramers formed by a combination of five subunits (GluK1-GluK5). GluK1, K2, and K3 are low-affinity subunits, while GluK4 and K5 are high-affinity subunits. Low-affinity subunits are able to form a functional receptor while high-affinity subunits need to form a complex with low-affinity subunits in order to produce a functional receptor (Pahl et al., 2014). KA receptors mediate postsynaptic depolarization, modulate synaptic release of GABA and glutamate, and play a role in the maturation of neural circuits during development. KA receptors become activated following presynaptic inhibition of GABA release. Interestingly, unlike NMDA and AMPA receptors, KA receptors activate a non-conventional metabotropic signaling pathway involving G proteins and second messengers. Dysfunction of KA receptor has been implicated in several CNS diseases

including schizophrenia, epilepsy, autism, depression, bipolar disorders, Huntington's disease, and mental retardation (Lerma and Marques, 2013).

I.4.1.4. Metabotropic glutamate receptors

mGluRs are members of the G-protein-coupled receptor (GPCR) superfamily. mGluRs are not ion channels. Instead, upon glutamate binding, G-protein is activated initiating an intracellular cascade that is important for the modulation of synaptic plasticity. There are eight mGluR subtypes which are classified into three groups: Group I includes mGluR1 and mGluR5. Group II includes mGluR2 and mGluR3. Finally, group III includes mGluR4 and mGluR6-8 (Lee et al., 2004). Group I is involved in phospholipase C activation, ERK kinase phosphorylation and, in some cases, the stimulation of adenyl cyclase. Activation of this group increases during neuronal excitability. Group II and III are involved in the inhibition of adenyl cyclase and Ca^{2+} channels and the activation of K^{+} channels. These groups are implicated in the induction of long-term depression (LTD) (Niswender and Conn, 2010). It has been proposed that the use of either agonists for mGluRs II and III or an antagonist for mGluA I may have protective effects (Nicoletti et al., 1996). Dysfunction of mGluRs has been reported in different types of neurodegenerative diseases such as ALS, multiple sclerosis, and Alzheimer's disease. In the retina, all mGluRs are expressed presynaptically, where they are important modulators of synaptic transmission. mGluR6 plays particularly important role in the retina as it mediates feedforward transmission from photoreceptors to ON bipolar cells. (Dhingra and Vardi, 2012). Elevated IOP increased mRNA levels of mGluR1a, mGluR2, mGluR4a, mGluR4b, mGluR6 and mGluR7a, while mGluR5a/b and mGluR8a mRNA levels become decreased (Dyka et al., 2004). In addition, the combination of MK801, a non-selective antagonist of NMDA, with LY354740, an agonist of the group II mGluRs, leads to partial

RGC protection in a rat model of glaucoma (Guo et al., 2006). These results suggest that the mGluR group II might contribute to RGC death.

I.4.2. Link between neuroinflammation and excitotoxicity.

Emerging evidence suggests that cytokines modulate ionotropic glutamate receptors. In fact, high levels of TNFα and IL-1β lead to the enhancement of glutamate transmission and neurodegeneration. Glial cells and neurons are essential for this process to occur (Fig. 8). Firstly, microglia release TNFα, which further stimulates TNFα release as well as glutamate release from themselves and astrocytes. Increase extracellular glutamate also stimulates TNFα release in microglia (Taylor et al., 2005). Second, astrocytes and Müller cells express high-affinity glutamate transporters (GLAST) (Ju et al., 2014). GLAST expression is reduced by TNFα (Tilleux and Hermans, 2007). Furthermore, TNFα stimulates glutamate release, resulting in increased levels of extracellular glutamate. Lastly, TNFα increases the synaptic excitatory/inhibitory ratio, ultimately resulting in excitotoxic cell death. TNFα acts on TNFR1 to increase the synaptic expression of GluA2-lacking AMPA receptors (Olmos and Llado, 2014; Viviani et al., 2014). This process is mediated by phosphatidylinositol 3-kinase (PI3-K) (Stellwagen et al., 2005). This mechanism was later confirmed in an excitotoxicity model. Intravitreal injection of NMDA leads to TNFα upregulation by Müller cells, mediating the surface expression of GluA2-lacking AMPA receptor (Lebrun-Julien et al., 2009).

IL-1β, which has been involved in the neuroinflammatory response, appears to also participate in glutamate-dependent excitotoxicity (Fogal and Hewett, 2008). Indeed, NMDA-induced excitotoxicity leads to over expression of IL-1β by microglia and astrocytes in the cortex and striatum of rats (Pearson et al., 1999). NMDA receptor antagonists result in down-

regulation of IL-1β following cerebral ischemia (Jander et al., 2000). In addition, exogenous IL-1β leads to an accumulation of intracellular Ca^{2+} through NMDA receptors. Neuronal death results from the activation of the Src family kinases and the phosphorylation of GluN2B (Viviani et al., 2003). IL-1β dysfunction has been suggested to contribute to several neurological disorders including Huntington's disease, Parkinson's disease, ALS, multiple sclerosis, HIV-associated dementia, and Alzheimer's disease (Fogal and Hewett, 2008). In the retina, IL-1β mediates increased glutamate uptake by Müller cells through a mechanism that involves increased $Na^{+/}K^{+}$ ATPase localization, in a model of NTG (Namekata et al., 2009). Collectively, these findings indicate that IL-1β plays contradicting roles depending on the cell type and experimental conditions. Therefore, more research is needed to better understand the interaction between IL-1β and glutamate excitotoxicity in CNS disorders.

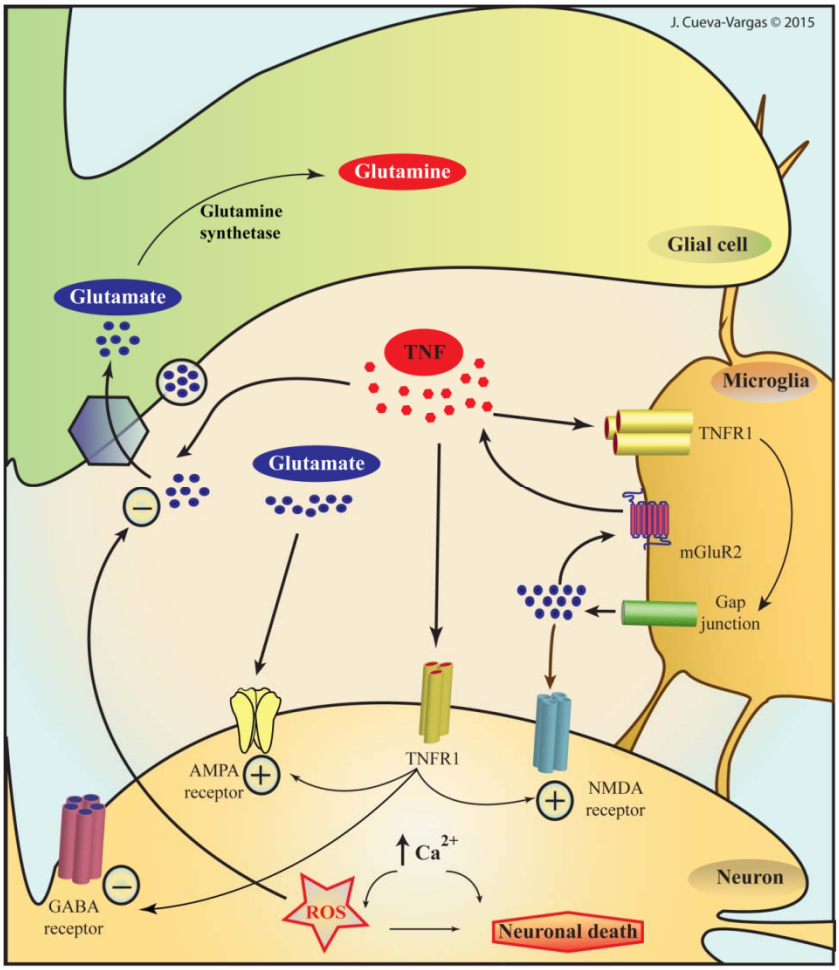


Figure 8. Schematic model showing the link between neuroinflammation and excitotoxicity. Early after induction of ocular hypertension, TNFα is released by Müller cells. In microglia, TNFα-mediated TNFR1 signaling promotes additional TNFα release and also glutamate release from hemichannels at gap junctions. In neurons, TNFα/TNFR1 binding increases the excitatory synaptic strength by inducing CP-AMPA receptors and/or NMDA receptors and decreasing the surface expression of GABA receptors. High levels of Ca^{2+} promote neuron death and excessive ROS, disrupting glutamate transport in neighboring glial cells. Source: Jorge Luis Cueva Vargas. Modified from: Copyright © 2014 Gabriel Olmos and Jerònia Lladó. Creative Commons Attribution License.

I.5. Current treatment and neuroprotective strategies for glaucoma

The standard current treatments for glaucoma focus primarily on the reduction of IOP. While this treatment is effective in some patients, delaying disease progression, IOP reduction is not always effective. There has been great interest in finding agents to promote neuroprotection to prevent or slow down the loss of RGCs and the progressive degeneration of the optic nerve. In addition, there is much interest in developing drugs to enhance blood flow to the retina and optic nerve. To date, there are no neuroprotective drugs clinically approved for use in glaucoma patients. Memantine, an NMDA open-channel receptor, was shown to have neuroprotective effects in a non-human primate glaucoma model (Hare et al., 2004; Yucel et al., 2006). However, in clinical trials, memantine failed to have a protective effect in glaucoma patients when compared to placebo-treated patients (Danesh-Meyer, 2011). In the following sections, I will discuss current treatments and recent approaches used for glaucoma.

I.5.1. Pharmacotherapy and regulation of IOP

Current glaucoma medications, which exert their effect by lowering IOP are grouped into five classes: beta-blockers, prostaglandins analogs, alpha-agonists, carbonic anhydrase inhibitors, and myotics. Beta blockers decrease the IOP by reducing aqueous humor production (Hayreh et al., 1999), however, their side effects cause exacerbation of respiratory symptoms, therefore they are not recommended for patients with asthma (Schwartz and Budenz, 2004). Non-selective beta-blockers include timolol and levobunolol. Betaxolol is a beta1-selective beta blocker (Vetrugno et al., 2008). Prostaglandins decrease the IOP by increasing the aqueous humor outflow through the uveoscleral pathway. In this pathway, the aqueous humor that passes from the anterior chamber through the uveal meshwork diffuses

through intercellular spaces of the ciliary body (Llobet et al., 2003). The side effects of prostaglandins include hyperemia, burning, tearing, itching, and changes in iris pigmentation. This group includes latanoprost, travoprost and bimatoprost (Vetrugno et al., 2008). Alpha-agonists include brimonidine, which is an α_2-adrenoceptor agonist that lowers IOP by reducing aqueous humor production and increasing outflow through the uveoscleral pathway (Adkins and Balfour, 1998). Side effects include dry mouth, systemic hypotension, fatigue and conjunctival blanching (Vetrugno et al., 2008). Carbonic anhydrase inhibitors lower IOP by decreasing the production of aqueous humor. This group includes dorzolamide, brinzolamide and the side effects include allergic reactions such as uticaria, angioderma or pruritus (Schmidl et al., 2015). Lastly, myotics include pilocarpine, aceclidine, acetylcholine, and carbachol. Since they are not well tolerated by many patients and are contraindicated for individuals younger than 40, they are not frequently used (Vetrugno et al., 2008).

I.5.2. Laser approaches

Laser surgery is another method to decrease the IOP. For the treatment of POAG, laser trabeculoplasty is used most frequently and successfully reduces IOP in 20-30% of glaucoma cases. The laser spots dilate and elongate the trabecular meshwork and/or Schlemm's canal (Wise and Witter, 1979). This technique is recommended for patients who do not tolerate pharmacologically-based IOP modification, as well as patients with pseudoexfoliative glaucoma and pigmentary glaucoma (Scuderi and Pasquale, 2008). Selective laser trabeculoplasty is another technique in which pigmented cells of the trabecular meshwork are selectively lysed without causing damage to the adjacent non-pigmented cells. This technique often produces better results than laser trabeculoplasty in eyes with non-pigmented angles (Schwartz and Budenz, 2004).

For patients with PACG, laser iridotomy, in which a hole is created in the iris to allow better flow of the aqueous humor, is an effective alternative treatment option. In 70-85% of treated patients, IOP was successfully reduced for one to three years without the use of additional drug therapy. Laser iridoplasty or gonioplasty is recommended when patients show plateau iris syndrome in which the iridocorneal angle is very narrow. This treatment is also highly effective and safe. In fact, 85-90% of patients with plateau iris syndrome retain an open angular recess for 10 years after a single surgery (Ritch et al., 2004; Scuderi and Pasquale, 2008). Finally, laser cyclophotocoagulation is considered a last resort glaucoma treatment in which non-pigmented epithelial cells of the ciliary body are destroyed to reduce IOP. This technique is performed either from inside the eye or through the sclera without opening the globe (Dietlein et al., 2009). It was recently reported to be useful in lowering IOP at any stage of the disease (Seibold et al., 2015)

I.5.3. Gene therapies

Gene therapy involves the replacement of a defective gene with a functional gene, the supplementation of a deficient gene, or the introduction of genetic material to reduce gene expression (gene silencing). Gene therapy is based on the introduction of DNA material to treat or prevent a disease by selectively targeting the affected cells. In glaucoma, gene therapy has focused on promoting RGC survival and decreasing IOP (Demetriades, 2011). The strategy of delivery includes the use of viral vectors, non-viral synthetic vectors, hybrid synthetic-viral systems, and the delivery of genetically manipulated cells. Among them, the use of viral vectors has been extensively used in preventing RGC death. The most commonly used viral vectors include adeno-associated virus (AAV), adenovirus and lentivirus vectors.

I.5.3.1. Adeno-associated virus

AAV are non-enveloped single-stranded DNA viruses belonging to the *parvoviridae* family. AAVs require a helper virus for replication. AAVs are generally considered to be non-pathogenic and non-toxic (Harvey et al., 2009), and can mediate long-term transgene expression, sometimes lasting for several years in the retina (Stieger et al., 2008). In addition, a new generation of self-complementary AAV (scAAV), which can be injected into the anterior chamber, has been reported to confer stable long term-expression of a reporter gene in rats and non-human primates (Buie et al., 2010). A disadvantage of AAV has been the limit in the size of the gene to be packaged, which is presently limited to 4.7 kb. Another limitation includes the laborious work needed to produce AAV vectors. Different AAV serotypes have been used to transduce cells into the retina, but AAV serotype 2 displays high tropism for RGCs after intravitreal injection (Cheng et al., 2002; Harvey et al., 2002). BDNF is one of the most potent pro-survival neurotrophic factor for injured RGCs. A pioneer study showed that intravitreal injection of an AAV vector encoding the BDNF receptor tropomyosin receptor kinase B (TrkB) in combination with exogenous BDNF promoted effective and long-term RGC survival after optic nerve injury (Cheng et al., 2002). In an ocular hypertension model, an AAV encoding BDNF promoted RGC survival one month after IOP elevation (Martin et al., 2003). AAV has also been used to deliver other trophic molecules including CNTF that promotes RGC survival and axonal regrowth after optic nerve crush (Leaver et al., 2006). Other neurotrophic factors delivered using gene therapy include GDNF (Schmeer et al., 2002) and fibroblast growth factor (FGF-2) (Sapieha et al., 2003).

I.5.3.2. Adenovirus

Adenovirus (Ad) is a linear double-stranded non-enveloped DNA virus. The advantages of using Ad include the ability to infect cells in different stages, to achieve high levels of foreign gene expression, and the relative ease to produce high titers Ad stocks (Liu et al., 2009). The main disadvantage of Ad virus, especially first generation vectors, is that it can result in severe complications including a severe inflammatory reaction and cytotoxic effect that limit their usefulness for longer periods (Isenmann et al., 2004). New modified Ad vectors have been designed, which are less immunogenic and provide long-term transfer of genetic material (Lamartina et al., 2007). Intravitreal injection of Ad vectors encoding BDNF (Di Polo et al., 1998), CNTF (Weise et al., 2000) or GDNF (Schmeer et al., 2002) have been shown to protect RGCs. In these studies Müller cells were also consistently transduced, suggesting that indirect trophic factors also contribute to the effect on RGC survival.

I.5.3.3. Lentivirus

Lentiviruses are a type of retrovirus that contains the necessary cellular and molecular machinery required to achieve efficient and stable gene expression in both dividing and non-dividing cells. The viral RNA is converted into double-stranded DNA by the reverse transcriptase and then transported to the nucleus. These vectors have large transgene carrying capacities compared to AAV. Also, their stable integration allows for long-term expression. Lentiviral vectors are derived from the primate lentiviruses HIV-1, HIV-2, and simian immunodeficiency virus. Vectors can also be generated from non-primate lentiviruses such as feline immunodeficiency virus, bovine immunodeficiency virus, and equine infectious anemia virus (Greenberg et al., 2006; Balaggan and Ali, 2012). The HIV based vectors have three

genes which are essential for gene delivery (*gag*, *pol* and *rev*), which are flanked by long-terminal repeats. Strategies for the treatment of glaucoma using lentivirus vectors include the ability to increase aqueous outflow through the drainage pathways in the trabecular meshwork. In this context, lentiviruses have shown high transduction efficiency of trabecular meshwork cells. Recently, an HIV-based lentivirus encoding enhanced green fluorescent protein (EGFP) gene was delivered into human trabecular meshwork cells with 92.3% efficiency *in vitro* and 88.16% efficiency *ex vivo*. These results suggest that HIV-based lentivirus have high potential for modification of trabecular meshwork gene expression, and consequently aqueous humor outflow, that might be useful for IOP regulation (Xiang et al., 2014).

I.5.4. Stem cells

Stem cells are defined as cells with an intrinsic ability to self-renew indefinitely and to differentiate into a variety of cell types. There are two sources of potential stem cells: i) embryonic stem cells, derived from the blastocysts and from the fetal retina, and ii) adult tissue-derived stem cells including: retinal stem cells from the ciliary epithelium, Müller stem cells, hippocampal stem cells, mesenchymal stem cells, oligodendrocyte precursor cells, olfactory ensheathing cells, and induced pluripotent stem cells (Sun et al., 2015). The use of stem cells should ideally provide chronic neuroprotection for a number of neurodegenerative diseases after a single treatment. However, hurdles in the implementation of stem cell-based strategies for brain diseases still need to be overcome to make the use of stem cells a clinical reality. Nonetheless, several studies have demonstrated that stem cell transplantation leads to synthesis and release of neurotrophic factors and results in immunomodulation (Bull et al., 2008). For example, in a rat model of glaucoma, mesenchymal stem cells were able to rescue RGCs by the secretion of growth factors and the modulation of the inflammatory process

(Emre et al., 2015). In addition, when neural stem cells genetically modified to secrete CNTF were differentiated into astrocytes, there was a significant reduction in the loss of axotomized RGCs (Flachsbarth et al., 2014). Cell-based therapies have also focused on re-establishing a healthy IOP. In fact, human induced pluripotent stem cells have been used to replace trabecular meshwork cells in an *in vitro* anterior segment organ culture and restored normal IOP levels (Abu-Hassan et al., 2015). Although this is a promising therapy for patients with adequate IOP elevation and visual impairment, it is not yet recommended due to limited knowledge regarding their safety (Sun et al., 2015).

I.5.5 Neuroprotection independent of IOP modulation

New drugs are developed based on our understanding of the mechanisms involved in the pathogenesis of the disease. Pharmacological neuroprotection, independent of IOP, represents an interesting alternative in order to prevent further progression of RGC death. During the last two decades, many experiments in animal glaucoma models have successfully identified potential targets for neuroprotection. Although these experiments show promising results, they cannot easily be transferred into clinical practice. These experiments include inhibiting apoptosis by blocking specific caspases, inhibiting excitotoxicity, increasing the availability of trophic factors, inhibiting acetylcholinesterase activity, maintaining normal mitochondria function, inhibiting oxidative stress, and finally, attenuating gliosis and inflammatory response (Osborne, 2008; Almasieh et al., 2010; Schmidl et al., 2015).

I.6. Objectives of the thesis, hypothesis and experimental approaches.

Problem and objectives: Glaucoma is the leading cause of irreversible blindness worldwide, affecting more than 70 million people. The disease is characterized by progressive

optic nerve degeneration and RGC death, resulting in visual field loss and blindness. Several risk factors have been proposed to contribute to the disease including elevated intraocular pressure, age, and genetic background. However the mechanism by which these risk factors promote RGC death is not well understood. At present, treatments for glaucoma are limited to lowering the IOP by medication or surgery. However, a large number of patients continue to experience optic nerve degeneration and vision loss in spite of responding well to treatment that regulate IOP. Therefore, understanding the mechanisms leading to RGC death in glaucoma is essential to develop new therapeutic approaches for the treatment and management of this disease. The main objectives of this thesis are:

i) To assess the cellular source of TNFα in the retina and changes in gene and protein expression in a rat model of ocular hypertension glaucoma.

ii) To characterize the role of soluble TNFα on RGC death and to establish its potential link with CP-AMPARS in experimental glaucoma.

iii) To establish whether anti-inflammatory therapies are effective at promoting RGC survival in glaucoma. Specifically, the role of ibudilast, an inhibitor of PDE4, on the response of IOP-dependent RGC survival will be explored.

Hypothesis: An important neuroinflammatory component has been proposed to mediate RGC death in glaucoma. TNFα, the most important pro-inflammatory cytokine, has been shown to play a key role in the development of an inflammatory reaction contributing to neuronal death. Therefore, the principal hypothesis of my thesis is that glial-derived soluble TNFα plays a critical role in RGC death by stimulating surface expression of CP-AMPARs in experimental glaucoma. Secondary hypothesis were also tested and include: i) that the use of selective inhibitors of TNFα or CP-AMPARs would enhance the survival of RGCs in

experimental glaucoma, and ii) that ibudilast, an anti-inflammatory clinically approved drug, can mediate RGC protection by attenuating gliosis and by reducing expression of pro-inflammatory cytokines including TNFα.

Experimental approaches: To achieve my research objectives, several experimental approaches were used:

i) The use of a rat model of experimental glaucoma, which consists of increasing the IOP by injecting a hypertonic saline solution into the episcleral vein in Brown Norway rats.

ii) The visualization and quantification of RGC survival using retrograde labeling with fluorescent tracers applied to the superior colliculus.

iii) The delivery of pharmacological reagents by intraperitoneal or intravitreal injections was used to determine the functional relevance of signaling pathways and determine their neuroprotective effect.

iv) The visualization and quantification of endogenous proteins was assessed by western blot analysis of fresh retinas and immunohistochemistry of retinal cross sections.

v) The levels of gene expression and the presence of edited GluA2 in control and glaucomatous retinas was carried out by polymerase chain reaction (PCR) technique

vi) The expression and function of CP-AMPARs in retinal neurons was established using the cobalt permeability assay and single-cell electrophysiological techniques

vii) The isolation of retinal-derive microglia ($CD11b^{+}$) was carried out using fluorescence-activated cell sorting (FACS) and the presence of genes of interest was performed using real-time quantitative PCR.

CHAPTER 2

II. FIRST ARTICLE: "SOLUBLE TUMOR NECROSIS FACTOR ALPHA PROMOTES RETINAL GANGLION CELL DEATH IN GLAUCOMA VIA CALCIUM-PERMEABLE AMPA RECEPTOR ACTIVATION"

Journal of Neuroscience (2015) Sep 2;35(35):12088-102.

Soluble tumor necrosis factor alpha promotes retinal ganglion cell death in glaucoma via calcium-permeable AMPA receptor activation.

Abbreviated title: TNFα and AMPAR-induced retinal neuron death.

Jorge L. Cueva Vargas [1], Ingrid K. Osswald [2], Nicolas Unsain [3], Mark R. Aurousseau [2], Philip A. Barker [3], Derek Bowie [2], and Adriana Di Polo [1].

[1] Department of Neuroscience and University of Montreal Hospital Research Center (CRCHUM), University of Montreal, Montreal, QC H2X 0A9, Canada
[2] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC H3G 1Y6, Canada
[3] Centre for Neuronal Survival, Montreal Neurological Institute, Montreal, QC H3A 2B4, Canada.

Corresponding author: Adriana Di Polo, Ph.D.
Dept. of Neuroscience
University of Montreal
CRCHUM, 900 Rue Saint-Denis
Tour Viger, Room R09.720
Montreal, Quebec H2X 0A9, Canada

Number of pages: 42
Number of figures and tables: Figures (7), Tables (1)
Number of words: Abstract (179), Introduction (667), Discussion (1475)
Conflict of Interest: The authors declare no competing financial interests.

Acknowledgements: We thank Dr. David Szymkowski (Xencor Inc.) for providing XPro1595; and Drs. Ray Tesi (FPRT Bio Inc.) and Timothy Kennedy (McGill University) for helpful comments on the manuscript. This work was supported by a team grant from the Canadian Institutes of Health Research (CIHR-VIH-105439). A.D.P. is a Chercheur National of Fonds de recherche Québec-Santé (FRQS).

N.U.'s current affiliation: Laboratorio de Neurobiología, Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Córdoba (UNC), Friuli 2434, Córdoba (5016), Argentina.

II.1. ABSTRACT

Loss of vision in glaucoma results from the selective death of retinal ganglion cells (RGCs). Tumor necrosis factor α (TNFα) signaling has been linked to RGC damage, however, the mechanism by which TNFα promotes neuronal death remains poorly defined. Using an *in vivo* rat glaucoma model, we show that TNFα is upregulated by Müller cells and microglia/macrophages soon after induction of ocular hypertension. Administration of XPro1595, a selective inhibitor of soluble TNFα, effectively protects RGC soma and axons. Using cobalt permeability assays, we further demonstrate that endogenous soluble TNFα triggers the upregulation of Ca^{2+}-permeable AMPA receptor (CP-AMPAR) expression in RGCs of glaucomatous eyes. CP-AMPAR activation is not caused by defects in GluA2 subunit mRNA editing, but rather reflects selective downregulation of GluA2 in neurons exposed to elevated eye pressure. Intraocular administration of selective CP-AMPAR blockers promotes robust RGC survival supporting a critical role for non-NMDA glutamate receptors in neuronal death. Our study identifies glia-derived soluble TNFα as a major inducer of RGC death through activation of CP-AMPARs, thereby establishing a novel link between neuroinflammation and cell loss in glaucoma.

II.2. INTRODUCTION

Glaucoma is the leading cause of irreversible blindness worldwide affecting more than 60 million people (Tham et al., 2014). The disease is characterized by progressive optic nerve degeneration resulting in vision loss. A crucial element in the pathophysiology of all forms of glaucoma is the death of retinal ganglion cells (RGCs), the neurons that convey visual information from the retina to the brain. High intraocular pressure has been identified as a major risk factor for developing the disease, but the mechanism by which elevated pressure injures RGCs is unknown. There is no cure for glaucoma and the standard of care is to lower eye pressure by medication or surgery. Many patients, however, continue to experience visual field loss even when pressure lowering treatments are implemented (Caprioli, 1997; Georgopoulos et al., 1997). A better understanding of the mechanisms involved in glaucomatous neurodegeneration is, therefore, essential to develop effective therapeutics.

Recent data demonstrate a neuroinflammatory component in glaucoma characterized by upregulation of proinflammatory cytokines, most notably tumor necrosis factor α (TNFα) (Tezel, 2013; Soto and Howell, 2014). TNFα is a pleiotropic cytokine with physiological and pathological functions that include regulation of inflammation, innate immunity, cancer, synaptic function and neurogenesis (Marin and Kipnis, 2013). TNFα is produced as a 22 kDa membrane-bound precursor that is cleaved by ADAM-17 (a disintegrin and metalloproteinase-17) to release the soluble 17 kDa mature TNFα (Moss et al., 1997). Both the transmembrane and soluble forms of TNFα are active and play distinct biological roles (Alexopoulou et al., 2006). Soluble TNFα binds primarily to TNFR1 and mediates apoptosis and chronic inflammation (Holtmann and Neurath, 2004; Brambilla et al., 2011; Novrup et al., 2014), whereas membrane-bound TNFα displays higher affinity for TNFR2 and regulates immunity against pathogens, resolution of

inflammation and myelination (Pasparakis et al., 1996; Arnett et al., 2001; Canault et al., 2004; Olleros et al., 2005; Alexopoulou et al., 2006; Ierna et al., 2009). Consistent with this, mice expressing only transmembrane TNFα suppress the onset and progression of autoimmune demyelination while maintaining host defenses against bacterial infection, septic shock and pulmonary fibrosis (Mueller et al., 1999; Olleros et al., 2002; Alexopoulou et al., 2006; Oikonomou et al., 2006).

TNFα signaling is linked to RGC damage. Exogenous administration of TNFα promotes RGC loss and optic nerve degeneration (Nakazawa et al., 2006; Lebrun-Julien et al., 2009b) and RGCs lacking TNFR1 are protected from mechanical injury (Tezel et al., 2004). TNFR2 deficiency was associated with increased RGC loss during retinal ischemia (Fontaine et al., 2002), yet lack of TNFR2 has been reported to protect RGCs from ocular hypertension damage (Nakazawa et al., 2006). The differential affinity of soluble versus transmembrane TNFα for its receptors as well as the timing of injury-related TNFα signaling (Mac Nair et al., 2014) may underlie these disparate data. Recently, high-throughput characterization of the retinal proteome revealed prominent upregulation of TNFα/TNFR1 signaling in human glaucoma (Yang et al., 2011). Notably, TNFα levels are elevated in aqueous humor samples from glaucoma patients (Sawada et al., 2010; Balaiya et al., 2011) and TNFα gene polymorphisms are associated with primary open angle glaucoma (Xin et al., 2013). In spite of these correlative data, the mechanism by which TNFα promotes RGC death is poorly understood.

TNFα exerts homeostatic control of synaptic strength by regulating α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid receptor (AMPAR) trafficking in the CNS (Pribiag and Stellwagen, 2014). Administration of exogenous TNFα to hippocampal neuron cultures induces cell surface expression of GluA2-lacking AMPARs, which are calcium (Ca^{+2})-permeable

(Beattie et al., 2002; Ogoshi et al., 2005; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Moreover, TNFα-induced expression of Ca^{+2}-permeable-AMPARs (CP-AMPAR) exacerbates neuronal death during acute ischemia and excitotoxicity (Ferguson et al., 2008; Leonoudakis et al., 2008; Lebrun-Julien et al., 2009b; Yin et al., 2012). In this study, we investigated whether soluble TNFα is an effector of RGC death and its mechanism of action in a rat glaucoma model. Our data reveal that ocular hypertension stimulates production of glia-derived soluble TNFα which triggers RGC surface expression of CP-AMPAR contributing to neuronal death.

II.3. MATERIALS AND METHODS

II.3.1. Experimental animals

All procedures were performed in male Brown Norway rats (300–400 g; Charles River Laboratories International, Inc., St-Constant, QC, Canada) in compliance with the guidelines of the Canadian Council on Animal Care for the Use of Experimental Animals (http://www.ccac.ca). The number of animals used in each experiment is indicated in the corresponding figure legend and table.

II.3.2. Ocular hypertension

Unilateral elevation of intraocular pressure was induced by injection of hypertonic saline solution into an episcleral vein as previously described (Morrison model) (Morrison et al., 1997; Almasieh et al., 2010). Briefly, a plastic ring was applied to the ocular equator to confine the injection to the limbal plexus and a microneedle was used to inject 50 µl of sterile 1.85 M NaCl through an episcleral vein. Following injection, the plastic ring was removed and the eyes were examined to assess the extent to which the saline solution traversed the limbal microvasculature.

Polysporin ophthalmic ointment (Pfizer Canada Inc., Kirkland, QC) was applied to the operated eye and the animal was allowed to recover. Eye pressure measurements were taken from awake animals after corneal application of one drop of proparacaine hydrochloride (0.5%, Alcon Laboratories, Inc., Fort Worth, TX) using a calibrated tonometer (TonoPen XL; Medtronic Solan, Jacksonville, FL). The tonometer was held perpendicular to the corneal surface and ten consecutive readings per eye were taken and averaged. Intraocular pressure was measured every other day, at the same time, for the entire duration of the experiment. The mean and peak (maximum) intraocular pressure values for each eye were calculated and used to estimate the mean and peak pressure for experimental and control groups.

II.3.3. Drug delivery

The following compounds were injected into the vitreous chamber in a total volume of 5 µl using a Hamilton syringe adapted with a 32-gauge glass microneedle: XPro1595 (50 µg/µl, Xencor Inc., Monrovia, CA), GYKI 52466 (250 µM, Tocris, Bristol, UK), or Philantotoxin 343 (PhTx 343) (250 µM, Sigma, St Louis, MO). Control animals received sterile phosphate buffer (PBS) vehicle. The tip of the needle was inserted into the superior hemisphere of the eye at a ~45° angle through the sclera into the vitreous body to avoid retinal detachment or injury to eye structures. Surgical glue (Indermill, Tyco Health Care, Mansfield, MA) was used to seal the injection site. For electrophysiological recordings, XPro1595 (Xencor Inc.) was administered every other day by intraperitoneal injection at a concentration shown to inhibit soluble TNFα *in vivo* (10 mg/kg) (Brambilla et al., 2011; Barnum et al., 2014).

II.3.4. Quantification of RGC soma and axons

Prior to induction of ocular hypertension, RGCs were retrogradely labeled with the indocarbocyanine dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate;

Molecular Probes, Junction City, OR) prepared by dissolving crystals (3%) in 0.9% NaCl. The superior colliculus was exposed and a small piece of gelfoam (Pharmacia and Upjohn Inc., Mississauga, ON) soaked in tracer was applied to the surface. For RGC density counts, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA), the retinas were dissected out and flat-mounted on a glass slide with the ganglion cell layer side up. DiI-labeled RGCs were counted in three square areas at 1, 2 and 3 mm from the optic disc in each retinal quadrant for a total of 12 retinal areas encompassing a total area of 1 mm^2. Macrophages and microglia that incorporated DiI after phagocytosis of dying RGCs were excluded from our quantitative analysis (Lebrun-Julien et al., 2009a). For axon counts, animals received a transcardial injection of heparin (1000 u/kg) and sodium nitroprusside (10 mg/kg) followed by perfusion with 2% PFA and 2.5% glutaraldehyde in 0.1 M PBS. Optic nerves were dissected, fixed in 2% osmium tetroxide, and embedded in epon resin. Semi-thin sections (0.7 μm) were cut on a microtome (Reichert, Vienna, Austria) and stained with 1% toluidine blue. RGC axons were counted at 1 mm from the optic nerve head in five non-overlapping areas of each optic nerve section, encompassing a total area of 5,500 μm^2 per nerve. The five optic nerve areas analyzed included: one in the center of the nerve, two peripheral dorsal and two peripheral ventral regions. The total area per optic nerve cross-section was measured using Northern Eclipse image analysis software (Empix Imaging, Toronto, ON), and this value was used to estimate the total number of axons per optic nerve.

II.3.5. Retinal immunohistochemistry

Animals were perfused transcardially with 4% PFA and retinal cryosections (16 μm) were prepared. The following primary antibodies were added to the retinal sections in blocking solution and incubated overnight at 4°C: TNFα (0.4 μg/ml; Millipore, Temecula, CA), Cellular

Retinaldehyde-Binding Protein (CRALBP, 1 µg/ml; ThermoFisher Scientific, Waltham, MA), TNFR1 (2 µg/ml Hycult biotech), TNFR2 (1.5 µg/ml Antibodies on-line), RNA-Binding Protein with Multiple Splicing (RBPMS, 0.01 µg/ml, PhosphoSolutions, Aurora, CO), and GluA2 (6C4, 2 µg/ml, Millipore, Temecula, CA). Sections were washed and incubated with secondary antibodies: anti-rabbit IgG (1 µg/ml, Cy3, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or anti-mouse IgG (1 µg/ml, FITC, Sigma-Aldrich). Some animals were subjected to application of Fluorogold (2%, Fluorochrome, Englewood, CO) to the superior colliculus, as described above, for visualization of RGCs on retinal sections. For whole-mount immunostaining, retinas were incubated at -80°C for 15 min, then overnight at 4°C in blocking solution (2% normal donkey serum, 2% Triton X-100 in PBS) containing an antibody against ionized calcium-binding adaptor molecule 1 (Iba1, 1.5 µg/ml, Wako Pure Chemicals Industries, Ltd., Osaka, Japan) and TNFα (4 µg/ml, R&D Systems), followed by secondary donkey anti-rabbit IgG Alexa Fluor 594 and anti-mouse IgG Alexa Fluor 488 (6 µg/ml Molecular Probes). Retinas were mounted on glass slides using an antifade reagent (Invitrogen, Eugene, OR, USA) for visualization with a Zeiss AxioSkop 2 Plus microscope (Carl Zeiss, Canada).

II.3.6. Semi-quantitative RT-PCR and Q/R site editing assay

Total RNA was isolated from individual retinas using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) and oligo $(dT)_{20}$ primers. PCR was performed using the following primers (300 nM): TNFα forward: 5'-TCCCAACAAGGAGGAGAAGTTCCC-3', TNFα reverse: 5'-AATGGCAA ATCGGCTGACGGTG-3', TNFR1 forward: 5'- ACCCCGGCTTCAACCCCACT-3', TNFR1 reverse: 5'-GGGTCTGCAGTGTCAAGCCGT-3', TNFR2 forward: 5'-GTGCAGGCCCCACCG

CATTT-3', TNFR2 reverse: 5'-TGGGCCTCCGCTGTGACTCT-3', GluA2 forward: 5'-CTATTT CCAAGGGGCGCTGAT-3', GluA2 reverse: 5'-CAGTCCAGGATTACACGCCG-3', β-actin forward: 5'-CACCACTTTCTACAATGAGC-3′, and β-actin reverse: 5'-CGGTCAGGATCTTC ATGAGG-3′. To assess Q/R site editing, the GluA2 open reading frame containing the Q/R site was amplified using forward (5'-GAATGGTATGGTTGGAGAGC-3') and reverse (5'-CACTT TCGATGGGAGACAC-3') primers. The expected 527 bp amplicon was obtained using a forward nested primer (5'-GCACACTGAGGAATTTGAAG-3') and an identical reverse primer resulting in a smaller 254 bp product, which was purified (MinElute; Qiagen, Toronto, ON) and digested with *Bbvl* (New England Biolabs, Whitby, ON). The reaction products were separated on agarose gels and visualized using a GelDoc™ imaging system (Biorad, Hercules, CA). Densitometry was performed using ImageJ software (http://imagej.nih.gov) and the purified PCR products were sequenced (McGill University and Génome Québec Innovation Centre, QC).

II.3.7. Subcellular fractionation and western blot analyses

Whole retinas were isolated and homogenized in fractionation buffer: 250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA supplemented with protease inhibitors. Samples were centrifuged for 13 min at 8700 x g to separate the nuclear fraction. The supernatant was centrifuged for an additional 6 min and 40 sec at 43000 x g to separate the mitochondria and lysosomal fraction (pellet). The supernatant containing the total membrane and cytosolic fractions was further centrifuged for 1 hr at 110000 x g, the pellet was resuspended in fractionation buffer. Retinal samples not subjected to fractionation were homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM Na fluoride, 0.25% Na deoxycholate and 2 nM $NaVO_3$ supplemented with protease and phosphatase inhibitors. Samples

were separated in 7.5-15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Blots were incubated overnight at 4°C in blocking buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1 % Tween-20 and 5% bovine serum albumin) containing each of the following primary antibodies: TNFα (1 µg/ml R&D Systems), TNFR1 (2 µg/ml Hycult Biotech, Plymouth Meeting, PA), TNFR2 (1 µg/ml Antibodies on-line, Atlanta, GA), GluA2 (4 µg/ml; Abcam, Cambridge, MA), Na^+/K^+ ATPase (0.1 µg/ml, Thermo Scientific, Rockford, IL), glyceraldehyde 3 phosphate dehydrogenase (GAPDH, 0.5 µg/ml, Cedarlane, Burlington, ON) or β-actin (0.5 µg/ml; Sigma-Aldrich, Oakville, ON). Membranes were washed and incubated in peroxidase-linked anti-mouse or anti-rabbit secondary antibodies (0.5 µg/ml, GE Healthcare, Mississauga, ON). Blots were developed with a chemiluminescence reagent (ECL, Amersham Biosciences) and exposed to X-OMAT imaging film (Eastman Kodak, Rochester, NY). Densitometry was performed using ImageJ software on scanned autoradiographic films obtained from a series of three independent western blots each carried out using retinal samples from distinct experimental or control groups.

II.3.8. Cobalt permeability assay

Retinas were dissected out, cut in small pieces and incubated for 30 min in oxygenated assay buffer (5 mM KCl, 2 mM $MgCl_2$, 12 mM glucose, 20 mM bicarbonate, 139 mM sucrose, 57.5 mM NaCl and 0.75 mM $CaCl_2$). The tissue was then incubated in 5 mM $CoCl_2$ and 10 mM L-glutamic acid in the presence or absence of AMPA receptor blockers GYKI 52466 (40 µM, Tocris) or the polyamine derivative PhTx 343 (50 µM, Sigma, St Louis, MO, USA). Cobalt (Co^{2+}) was precipitated with 0.24% ammonium sulfide and the retina was then fixed in 0.8% glutaraldehyde and sections were prepared (20 µm). Silver enhancement of the Co^{2+} sulfide precipitate was performed with the Amersham Intense kit (GE Healthcare, Buckinghamshire,

UK), then retinal sections were rinsed, mounted and imaged with a Zeiss Axioplan 2 imaging microscope.

II.3.9. Statistical analyses

Data analysis and statistics were performed using the GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA), followed by the Tukey's Multiple Comparison *post hoc* test, or by a Student's *t* test as indicated in the legends.

II.4. RESULTS

II.4.1. TNFα and its receptors are rapidly upregulated in experimental glaucoma.

Unilateral ocular hypertension was induced in Brown Norway rats by a single injection of hypertonic saline into an episcleral vein as described (Almasieh et al., 2010; Almasieh et al., 2013). This procedure leads to blockade of aqueous humor outflow, gradual increase of eye pressure and selective loss of RGC soma and axons (Fig. 1A-C), producing a model that displays an excellent linear correlation between intraocular pressure and neuronal death. Inner retinal atrophy, optic nerve degeneration, and optic nerve head remodeling in this model are similar to those observed in human glaucoma (Morrison et al., 2005) therefore it is an excellent *in vivo* paradigm of glaucomatous damage.

We first asked whether there are variations in endogenous TNFα levels following glaucoma induction. Because early changes are more likely to play a causative role in RGC loss, our analysis focused on events at 1 and 3 weeks after hypertonic saline injection. Quantitative RT-PCR of retinal samples demonstrated a gradual and significant increase of TNFα mRNA in glaucomatous retinas compared to non-injured controls (intact) (Fig. 1D and E). Similarly, western blot analysis showed a marked increase in TNFα protein levels in retinas with high

intraocular pressure (Fig. 1F and G). Immunohistochemistry confirmed that while retinal TNFα was low in control non-injured eyes, it increased substantially with ocular hypertension (Fig 1H and I). Co-labeling experiments using cellular retinaldehyde binding protein (CRALBP), a Müller cell-specific marker, demonstrated that these glial cells upregulate TNFα soon after glaucoma induction (Fig. J-M). On flat-mounted retinas, analysis of the inner plexiform layer using Iba1, a pan-microglia and macrophage marker (Imai and Kohsaka, 2002), revealed reduced ramifications and increased soma size in Iba1-positive cells suggestive of an activated state during ocular hypertension (Fig. 1N, O). TNFα was not detected in highly ramified, presumably quiescent, Iba1-positive cells found in non-injured retinas, but increased markedly in cells with round somata and simplified processes (Fig. 1P-U).

We then asked whether the expression of TNFα receptors, TNFR1 and TNFR2, was altered in this glaucoma model. Our findings reveal a progressive increase of both TNFR1 and TNFR2 mRNA (Fig. 2A and B) and protein (Fig. 2C-F) detected as early as 1 week after induction of ocular hypertension. Retinal immunohistochemistry confirmed an increase in TNFR1- and TNFR2-positive cells (Fig. 2G and H). In the ganglion cell layer, TNFR1 and TNFR2 were upregulated in RGCs, visualized with the retrograde tracer Fluorogold (FG) (Fig. 2I and J). We conclude that high intraocular pressure rapidly stimulates TNFα production by retinal glia and increases TNFR1/2 expression in RGCs.

II.4.2. Inhibition of soluble TNFα promotes robust RGC soma and axon protection.

We next sought to test whether soluble TNFα contributes to RGC loss using XPro1595, a dominant-negative protein that selectively inhibits soluble TNFα without interfering with transmembrane TNFα or TNFR (Zalevsky et al., 2007). XPro1595 was injected intraocularly at 1 and 2 weeks after induction of ocular hypertension, and RGC survival was evaluated a week later

(3 weeks after injury onset). Cell soma and axon quantification was performed blinded to treatment. Flat-mounted retinas from eyes treated with XPro1595 showed higher densities of DiI-positive RGCs compared to control retinas treated with vehicle (PBS) (Fig. 3A-C). Quantitative analysis demonstrated that XPro1595 promoted significant RGC survival (94%: 1785 ± 27 RGCs/mm^2, mean ± S.E.M., n=7) with respect to eyes that received vehicle (65%: 1244 ± 55 RGCs/mm^2, n=6) (Fig. 3G).

A hallmark of glaucoma is the degeneration of RGC axons in the optic nerve posterior to the lamina cribrosa; therefore, we also investigated the capacity of XPro1595 to protect axons. Analysis of optic nerve cross-sections showed a substantially larger number of RGC axon fibers with normal morphology in XPro1595-treated eyes compared to vehicle-treated controls (Fig. 3D-F). The latter displayed extensive axon degeneration including disarray of fascicular organization and degradation of myelin sheaths. Quantitative analysis confirmed that XPro1595 promoted marked protection of RGC axons (80%: 79917 ± 1574 axons/nerve, mean ± S.E.M., n=6) compared to vehicle treatment (45%: 45345 ± 7583 axons/nerve, n=6) (Fig. 3H).

To assess whether XPro1595 reduced intraocular pressure, which could account for its neuroprotective effect, we measured eye pressure every other day after glaucoma induction. Our results show that XPro1595 administration did not reduce eye pressure and that the pressure elevations among XPro1595-treated and vehicle-treated groups were similar (Table 1). Given that the rate of RGC death is proportional to ocular hypertension, the similar increase in intraocular pressure amongst groups allowed for reliable comparison of the neuroprotective effect of XPro1595 versus vehicle. Our results demonstrate that XPro1595 protects both RGC soma and axons, without altering eye pressure, suggesting a prominent role for soluble TNFα in glaucoma downstream ocular hypertension.

II.4.3. Ocular hypertension-induced TNFα triggers CP-AMPAR activity in RGCs.

To investigate whether soluble TNFα modulates CP-AMPAR in glaucoma, we used an *in situ* cobalt (Co^{2+}) permeability assay (Aurousseau et al., 2012). Co^{2+} is transported into neurons expressing CP-AMPAR in the plasma membrane, but it does not permeate through other Ca^{2+} channels or NMDA receptors (Hagiwara and Byerly, 1981; Mayer and Westbrook, 1987). Following Co^{2+} precipitation, the appearance of a dark brown color is visualized only in neurons containing CP-AMPAR. In non-injured retinas, Co^{2+} accumulation was detected solely in cells of the inner nuclear layer (INL, Fig. 4A), previously identified as horizontal and AII amacrine cells (Osswald et al., 2007). Remarkably, ocular hypertension induced massive Co^{2+} accumulation in the ganglion cell layer (GCL), where RGC soma are located, and in the inner plexiform layer (IPL), where dendrites are present (Fig. 4B). Displaced amacrine cells account for ~40-50% of the total number of neurons in the GCL (Perry, 1981; Schlamp et al., 2013). Hence, we performed co-labeling with the RGC-specific marker 'RNA binding protein with multiple splicing' (RBPMS) (Kwong et al., 2010; Rodriguez et al., 2013) to selectively label RGCs in intact and glaucomatous retinas (Fig 4C and D). Co-localization of Co^{2+} precipitate with RBPMS confirmed that Co^{2+} accumulated in RGCs (Fig. 4E-G).

To test whether soluble TNFα was required for increased CP-AMPAR levels leading to Co^{2+} accumulation, XPro1595 was injected intraocularly 2 weeks after glaucoma induction and Co^{2+} staining was performed a week later. Figure 4H shows that XPro1595 strongly inhibited Co^{2+} uptake by RGCs. Treatment with GYKI 52466, a non-competitive AMPAR antagonist (Mellor, 2010), or Philantotoxin 343 (PhTx 343), a polyamine-derived compound that selectively antagonizes CP-AMPAR (Stromgaard et al., 2005), effectively blocked Co^{2+} uptake (Fig. 4I and J). These data indicate that Co^{2+} accumulation in RGCs was due to selective flux through CP-

AMPAR and rule out the contribution of other glutamate receptors. We conclude that, in glaucoma, soluble TNFα triggers the CP-AMPAR activity detected in RGCs.

II.4.4. Retinal AMPAR GluA2 subunit is fully edited at the Q/R site in glaucoma.

The Ca^{2+} permeability of AMPAR varies depending on whether the GluA2 subunit is present and, if so, whether it has undergone mRNA editing. GluA2 mRNA editing switches an uncharged amino acid glutamine (Q) to a positively-charged arginine (R) and, as a result, Ca^{2+} cannot be transported due to electrostatic repulsion by the arginine residues lining the AMPAR pore (Burnashev et al., 1992). We then asked whether GluA2 mRNA transcripts were edited as a mechanism accounting for AMPAR Ca^{2+} permeability in glaucoma. To this end, a 254-bp amplicon of rat GluA2 incorporating the Q/R site was amplified from cDNA generated by RT-PCR using retinal mRNA. Taking advantage of the presence of an additional *Bbvl* restriction site unique to the unedited GluA2 (Q) sequence, digestion of the PCR product resulted in a distinct band pattern consistent with GluA2 control plasmid coding for arginine at the Q/R site in both intact and glaucomatous retinas (Fig. 5A, 5B). In addition, parallel sequencing of the amplicon confirmed that GluA2 transcripts from both control and glaucoma samples encoded an arginine residue at the Q/R site (Fig. 5 C-F). Our data demonstrate that the GluA2 subunit is fully edited in glaucoma, and strongly suggest that GluA2 editing is not the cause for increased CP-AMPAR expression.

II.4.5. GluA2 expression in RGCs is downregulated by ocular hypertension.

Reduced expression or absence of the GluA2 subunit results in AMPARs that are permeable to Ca^{+2} (Dingledine et al., 1999). To determine whether TNFα-mediated loss of GluA2 could account for increased CP-AMPAR activity in RGCs, we examined GluA2 levels in glaucomatous retinas. Our results show that ocular hypertension triggers a gradual and

substantial decrease of both retinal GluA2 mRNA and protein (Fig. 6A-D). Subcellular fractionation of membrane and cytosolic fractions confirmed a significant reduction of membrane-associated GluA2 in hypertensive retinas (Fig. 6E and F). The enrichment of each fraction for membrane or cytosolic proteins was confirmed using Na^+/K^+ ATPase or GAPDH, respectively (Fig. 6E). To establish whether GluA2 was downregulated in RGCs, retinal immunofluorescence was performed. A striking reduction of GluA2 labeling was observed in the ganglion cell layer of retinas subjected to ocular hypertension (GCL, Fig. 6G and H). Co-staining of GluA2 with the RGC-specific marker RBPMS confirmed that loss of GluA2 labeling reflected protein downregulation and not death of RGCs (Fig. 6G and H). We conclude that RGC levels of GluA2 are markedly reduced in glaucoma.

II.4.6. CP-AMPAR blockade promotes RGC soma and axon survival.

To determine if TNFα mediates RGC death by modulating plasma membrane levels of CP-AMPAR, we examined the neuroprotective effect of AMPAR channel blockers. Our data show that a single injection of GYKI 52466 or PhTx 343 promoted RGC soma and axon protection at 3 weeks after ocular hypertension compared to vehicle-treated retinas (Fig. 7A-F). Quantitative analysis was performed blinded to treatment and confirmed that GYKI 52466 and PhTx 343 resulted in striking RGC soma survival (88% and 91%, respectively) compared to vehicle (65%) (Fig. 7G). Similarly, quantification of RGC axons in optic nerve cross sections revealed robust axonal protection by GYKI 52466 (70%) and PhTx 343 (73%) with respect to vehicle-treated controls (46%) (Fig. 7H). Consistent with the idea that the primary site of degeneration in glaucoma is at the level of the axon, we observed more pronounced axon loss than soma loss. Nonetheless, both GYKI 52466 and PhTx protected a similar proportion of RGC soma and axons. Our data demonstrate a critical role for CP-AMPAR in RGC death in glaucoma.

II.5. DISCUSSION

Despite data linking TNFα with glaucomatous neurodegeneration, little is known about the form of TNFα that promotes RGC death and its underlying mechanism of action. In this regard, the data presented here, using a well characterized rat glaucoma model reveal several novel findings. First, we show that ocular hypertension rapidly stimulates production of TNFα by Müller cells and microglia/macrophages, and increases TNFR1 and TNFR2 expression in RGCs. Second, we demonstrate that XPro1595, an inhibitor of soluble TNFα, effectively protects RGC soma and axons. Third, we show that TNFα stimulates the expression of CP-AMPAR in RGCs, a response that does not depend on defects in GluA2 mRNA editing but reflects selective downregulation of GluA2 by these neurons. Lastly, our results demonstrate that CP-AMPAR blockers promote robust RGC survival supporting a critical role for non-NMDA glutamate receptors in glaucomatous damage. Collectively, this study identifies a novel mechanism by which glia-derived soluble TNFα modulates neuronal CP-AMPA function leading to RGC death, and establishes a critical link between neuroinflammation and cell damage in glaucoma.

During glaucoma, chronically reactive glial cells are thought to become a sustained source of proinflammatory cytokines (Seitz et al., 2013). Our data demonstrate that Iba1-positive microglia/macrophages are a source of TNFα in ocular hypertensive eyes. TNFα expression was observed primarily in microglia/macrophages with amoeboid shape, characteristic of a reactive state, rather than in resting cells with ramified morphology. This finding is consistent with previous reports showing TNFα expression in microglia from human glaucomatous optic nerve head (Yuan and Neufeld, 2001) and rat retinas subjected to episcleral vein cauterization (Roh et al., 2012). Of interest, high-dose irradiation leading to reduced microglial activation, and

presumably decreased levels of proinflammatory mediators, attenuated RGC degeneration in a mouse model of inherited pigmentary glaucoma (Bosco et al., 2012; Howell et al., 2012). Moreover, our results demonstrate that Müller cells, the most abundant glial cell type in the retina, upregulate TNFα in response to increased eye pressure. Excitotoxic damage has also been shown to rapidly increase NFκB (nuclear factor kappa B) activation and TNFα production by Müller cells (Lebrun-Julien et al., 2009b). It is currently unknown how ocular hypertension stimulates TNFα output by reactive glia but it might involve mechanisms dependent on ischemia/hypoxia. For example, cultured microglia subjected to hypoxia release more TNFα and interleukin 1β than cells exposed to normal oxygen levels (Sivakumar et al., 2011). Accumulation of toxic factors may also increase glia reactivity and subsequent neuroinflammation. Indeed, we have previously shown that the precursor form of nerve growth factor activates neurotrophin receptor $p75^{NTR}$-dependent signaling mechanisms on Müller cells, leading to increased production of TNFα and subsequent RGC death (Lebrun-Julien et al., 2010).

The two active forms of TNFα, soluble and transmembrane, play distinct biological roles. We employed XPro1595 to block soluble TNFα and to elucidate its role in glaucomatous neurodegeneration. XPro1595 is an engineered protein that selectively binds with soluble TNFα monomers to form inactive heterotrimers unable to interact with TNFα receptors. Therefore, XPro1595 eliminates signaling from soluble TNFα without interfering with the transmembrane form (Steed et al., 2003; Zalevsky et al., 2007). Our data demonstrate that XPro1595 effectively promotes RGC survival, without altering intraocular pressure. Given that both TNFR1 and TNFR2 are upregulated by RGCs during ocular hypertension, we speculate that blockade of soluble TNFα minimizes the detrimental effect of TNFR1 activation while preserving beneficial

TNFR2-mediated signaling. Recent studies also reported a neuroprotective effect of XPro1595 in models of experimental autoimmune encephalomyelitis, spinal cord injury, Parkinson's and Huntington's disease, confirming a harmful role for soluble TNFα in neurodegenerative conditions (Brambilla et al., 2011; Barnum et al., 2014; Hsiao et al., 2014; Novrup et al., 2014). Etanercept, a drug that blocks both soluble and transmembrane TNFα, has been shown to protect RGCs in a rat glaucoma model (Roh et al., 2012). However, non-selective TNFα inhibitors, including etanercept, infliximab and adalimumab, have been linked to serious adverse effects such as impaired host defense, autoimmunity, lupus, demyelination syndromes and congestive heart failure (van Oosten et al., 1996; Shakoor et al., 2002; Slifman et al., 2003; Sfikakis, 2010; Keane et al., 2001; Lee et al., 2002). Collectively, these findings highlight the benefits of inhibiting soluble TNFα while preserving transmembrane TNFα function during neurodegeneration.

In physiological conditions, TNFα is an important modulator of synaptic plasticity by its ability to regulate AMPA receptor trafficking. In hippocampal pyramidal neurons, TNFα strengthens synapses by rapid exocytosis of AMPAR that lack or have low stoichiometric amounts of the GluA2 subunit thus enhancing intracellular Ca^{+2} rises (Ogoshi et al., 2005; Stellwagen et al., 2005). We found low levels of endogenous TNFα in the uninjured adult rat retina that correlated with sparse expression of CP-AMPAR. Using a Co^{2+} permeability assay applied to adult intact retinas, we confirmed that CP-AMPAR expression was restricted to inhibitory interneurons, horizontal and AII amacrine cells, as shown previously (Mørkve et al., 2002; Singer and Diamond, 2003; Veruki et al., 2003; Osswald et al., 2007). Although CP-AMPAR have been reported in acutely purified neonatal RGCs and can contribute to excitotoxicity (Park et al., 2015), CP-AMPAR have not been previously detected in adult RGCs.

Remarkably, we show that ocular hypertension triggers robust CP-AMPAR upregulation in these neurons. Our finding that XPro1595 attenuates Co^{2+} accumulation in RGCs indicates that soluble TNFα contributes to the increase of CP-AMPAR. Our data suggest that while basal levels of TNFα are required for retinal homeostasis and neurotransmission, excess soluble TNFα results in CP-AMPAR upregulation and cell death in glaucoma.

Despite the presence of CP-AMPAR in horizontal and AII amacrine cells, these neurons are not lost in glaucoma. Indeed, morphological alterations in amacrine and horizontal cells have been described in ocular hypertension models, but the general consensus is that these interneurons do not die (Kielczewski et al., 2005; Gunn et al., 2011; Vidal-Sanz et al., 2012; Fernández-Sánchez et al., 2014). Several factors may contribute to the susceptibility of RGCs to excitotoxicity via CP-AMPAR including poor cytosolic Ca^{2+} buffering leading to mitochondrial Ca^{2+} overload and generation of reactive oxygen species (Crish and Calkins, 2011). Although much emphasis has been placed on Ca^{2+}, other divalent cations that permeate through CP-AMPAR might also contribute to RGC loss. For example, AMPARs are highly permeable to zinc (Zn^{2+}), which can be extremely toxic for neurons (Sensi et al., 1999; Jiang et al., 2001; Kim and Koh, 2002; Sensi et al., 2003), and Zn^{2+} is known to rapidly accumulate in hippocampal neurons following ischemia (Koh et al., 1996; Lee et al., 2003). Recently, Zn^{2+} has been shown to play a role in oxidative stress and age-related neurodegeneration (McCord and Aizenman, 2014). The future elucidation of the role of Zn^{2+} in RGC death is of interest to understand its potential contribution to CP-AMPAR-mediated damage in glaucoma.

How do AMPAR become permeable to divalent ions in glaucoma? One possibility is defective GluA2 mRNA editing. Typically, the change from an uncharged amino acid glutamine (Q) to a positively charged arginine (R) in GluA2 is sufficient to confer Ca^{2+} impermeability

(Burnashev et al., 1992). Abnormal mRNA processing failing to complete this process will result in a Ca^{2+}-permeable AMPAR pore. Our findings show that retinal GluA2 is fully edited in glaucoma, ruling out a post-transcriptional editing defect as a mechanism by which AMPARs become divalent permeable. A second possibility is a decrease in GluA2 leading to AMPARs that are permeable to divalent ions. Using biochemical and immunohistochemical analyses, we demonstrate that GluA2 expression is substantially downregulated in RGCs subjected to ocular hypertension. GluA2-lacking CP-AMPAR are traditionally thought of as Ca^{2+} permeable channels that are blocked by exogenous and endogenous polyamines, while GluA2-containing receptors are impermeable to Ca^{2+} and lack polyamine sensitivity (Bowie et al., 1999; Dingledine et al., 1999). Of interest, a third class of AMPAR characterized by divalent permeability and weak sensitivity to polyamine block has been proposed to exist in some CNS regions including the retina (Bowie, 2012). Indeed, while divalent permeability through AMPAR in horizontal and AII amacrine cells was effectively blocked with the polyamine derivative PhTX 343 before eye opening, these AMPAR became insensitive to PhTX 343 by postnatal day 14 (Osswald et al., 2007). Our data show that PhTX 343 blocks divalent permeability through CP-AMPAR and, in doing so, promotes robust RGC survival in glaucomatous eyes. These observations suggest that adult retinal neurons exposed to ocular hypertension revert to an immature phenotype characterized by CP-AMPAR that are permeable to divalent cations and sensitive to polyamine block. Our findings also reveal the potential to use of polyamine-derived compounds as neuroprotective agents for glaucoma.

In conclusion, using multiple complementary techniques we demonstrate a crucial role of glia-derived soluble TNFα in glaucomatous neurodegeneration. Importantly, our data identify a novel and important mechanism of soluble TNFα-induced damage involving GluA2

downregulation and increased CP-AMPAR leading to RGC death. These findings expand our understanding of the molecular basis of RGC damage during neuroinflammation and might have implications in the design of neuroprotective strategies for glaucoma.

II.6. REFERENCES

Alexopoulou L, Kranidioti K, Xanthoulea S, Denis M, Kotanidou A, Douni E, Blackshear PJ, Kontoyiannis DL, Kollias G (2006) Transmembrane TNF protects mutant mice against intracellular bacterial infections, chronic inflammation and autoimmunity. Eur J Immunol 36:2768-2780.

Almasieh M, Zhou Y, Kelly MEM, Casanova C, Di Polo A (2010) Structural and functional neuroprotection in glaucoma: role of galantamine-mediated activation of muscarinic acetylcholine receptors. Cell Death Dis 1:e7.

Almasieh M, MacIntyre JN, Pouliot Mn, Casanova C, Vaucher E, Kelly MEM, Di Polo A (2013) Acetylcholinesterase inhibition promotes retinal vasoprotection and increases ocular blood flow in experimental glaucoma. Invest Ophthalmol & Vis Sci 54:3171-3183.

Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JPY (2001) TNF[alpha] promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci 4:1116-1122.

Aurousseau MRP, Osswald IK, Bowie D (2012) Thinking of Co2+-staining explant tissue or cultured cells? How to make it reliable and specific. Eur J Neurosci 35:1201-1207.

Balaiya S, Edwards J, Tillis T, Khetpa lV, Chalam KV (2011) Tumor necrosis factor-alpha (TNF-α) levels in aqueous humor of primary open angle glaucoma. Clin Ophthalmol 5:553-556.

Barnum CJ, Chen X, Chung J, Chang J, Williams M, Grigoryan N, Tesi RJ, Tansey MG (2014) Peripheral administration of the selective inhibitor of soluble tumor necrosis factor (TNF) XPro1595 attenuates nigral cell loss and glial activation in 6-OHDA hemiparkinsonian rats. J Parkinson's Dis 4:349-360.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Bosco A, Crish SD, Steele MR, Romero CO, Inman DM, Horner PJ, Calkins DJ, Vetter ML (2012) Early reduction of microglia activation by irradiation in a model of chronic glaucoma. PLoS ONE 7:e43602.

Bowie D (2012) Redefining the classification of AMPA-selective ionotropic glutamate receptors. J Physiol 590:49-61.

Bowie D, Bähring R, Mayer ML (1999) Block of kainate and AMPA receptors by polyamines and arthropod toxins. In: Handbook of Experimental Pharmacology; Ionotropic Glutamate Receptors in the CNS (H. JPaM, ed), pp 251-373. Berlin: Springer-Verlag.

Brambilla R, Ashbaugh JJ, Magliozzi R, Dellarole A, Karmally S, Szymkowski DE, Bethea JR (2011) Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination. Brain 134:2736-2754.

Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8:189-198.

Canault M, Peiretti F, Mueller C, Kopp F, Morange P, Rihs S, Portugal H, Juhan-Vague I, Nalbone G (2004) Exclusive expression of transmembrane TNF-α in mice reduces the inflammatory response in early lipid lesions of aortic sinus. Atherosclerosis 172:211-218.

Caprioli J (1997) Neuroprotection of the optic nerve in glaucoma. Acta Ophthalmol Scand 75:364-367.

Crish SD, Calkins DJ (2011) Neurodegeneration in glaucoma: progression and calcium-dependent intracellular mechanisms. Neurosci 176:1-11.
Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51:7-62.
Ferguson AR, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS (2008) Cell death after spinal cord injury is exacerbated by rapid TNF{alpha}-induced trafficking of GluR2-lacking AMPARs to the plasma membrane. J Neurosci 28:11391-11400.
Fernández-Sánchez L, de Sevilla Müller LP, Brecha NC, Cuenca N (2014) Loss of outer retinal neurons and circuitry alterations in the DBA/2J mouse. Invest Ophthalmol & Vis Sci 55:6059-6072.
Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U (2002) Neurodegenerative and Neuroprotective Effects of Tumor Necrosis Factor (TNF) in Retinal Ischemia: Opposite Roles of TNF Receptor 1 and TNF Receptor 2. J Neurosci 22:216RC-.
Georgopoulos G, Andreanos D, Liokis N, Papakonstantinou D, Vergados J, Theodossiadis G (1997) Risk factors in ocular hypertension. Eur J Ophthalmol 7:357-363.
Gunn DJ, Gole GA, Barnett NL (2011) Specific amacrine cell changes in an induced mouse model of glaucoma. Clin Experiment Ophthalmol 39:555-563.
Hagiwara S, Byerly L (1981) Calcium Channel. Ann Rev Neurosci 4:69-125.
Holtmann M, Neurath M (2004) Differential TNF-signaling in chronic inflammatory disorders. Curr Mol Med 4:439-444.
Howell GR, Soto I, Zhu X, Ryan M, Macalinao DG, Sousa GL, Caddle LB, MacNicoll KH, Barbay JM, Porciatti V, Anderson MG, Smith RS, Clark AF, Libby RT, John SWM (2012) Radiation treatment inhibits monocyte entry into the optic nerve head and prevents neuronal damage in a mouse model of glaucoma. J Clin Invest 122:1246-1261.
Hsiao H-Y, Chiu F-L, Chen C-M, Wu Y-R, Chen H-M, Chen Y-C, Kuo H-C, Chern Y (2014) Inhibition of soluble tumor necrosis factor is therapeutic in Huntington's disease. Hum Mol Genet 23:4328-4344.
Ierna MX, Scales HE, Mueller C, Lawrence CE (2009) Transmembrane tumor necrosis factor alpha is required for enteropathy and is sufficient to promote parasite expulsion in gastrointestinal helminth infection. Infection and Immunity 77:3879-3885.
Imai Y, Kohsaka S (2002) Intracellular signaling in M-CSF-induced microglia activation: Role of Iba1. Glia 40:164-174.
Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH (2001) Zn2+ Induces Permeability Transition Pore Opening and Release of Pro-apoptotic Peptides from Neuronal Mitochondria. J Biol Chem 276:47524-47529.
Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 345:1098-1104.
Kielczewski JL, Pease ME, Quigley HA (2005) The Effect of Experimental Glaucoma and Optic Nerve Transection on Amacrine Cells in the Rat Retina. Invest Ophthalmol & Vis Sci 46:3188-3196.
Kim Y-H, Koh J-Y (2002) The Role of NADPH Oxidase and Neuronal Nitric Oxide Synthase in Zinc-Induced Poly(ADP-ribose) Polymerase Activation and Cell Death in Cortical Culture. Exp Neurol 177:407-418.

Koh J, Suh S, Gwag B, He Y, Hsu C, Choi D (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013-1016.
Kwong JMK, Caprioli J, Piri N (2010) RNA Binding Protein with Multiple Splicing: A New Marker for Retinal Ganglion Cells. Invest Ophthalmol & Vis Sci 51:1052-1058.
Lebrun-Julien F, Morquette B, Douillette A, Saragovi HU, Di Polo A (2009a) Inhibition of p75NTR in glia potentiates TrkA-mediated survival of injured retinal ganglion cells. Mol Cell Neurosci 40:410-420.
Lebrun-Julien F, Bertrand M, De Backer O, Stellwagen D, Morales CR, Di Polo A, Barker PA (2010) ProNGF induces TNFα-dependent death of retinal ganglion cells through a p75NTR non-cell-autonomous signaling pathway. Proc Natl Acad Sci U S A 107:3817-3822.
Lebrun-Julien F, Duplan L, Pernet V, Osswald IK, Sapieha P, Bourgeois P, Dickson K, Bowie D, Barker PA, Di Polo A (2009b) Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. J Neurosci 29:5536-5545.
Lee J-Y, Kim J-H, Palmiter RD, Koh J-Y (2003) Zinc released from metallothionein-iii may contribute to hippocampal CA1 and thalamic neuronal death following acute brain injury. Exp Neurol 184:337-347.
Lee JH, Slifman NR, Gershon SK, Edwards ET, Schwieterman WD, Siegel JN, Wise RP (2002) Life-threatening histoplasmosis complicating immunotherapy with tumor necrosis factor alpha antagonists infliximab and etanercept. Arthritis Rheum 46:2565-2570.
Leonoudakis D, Zhao P, Beattie EC (2008) Rapid Tumor Necrosis Factor {alpha}-Induced Exocytosis of Glutamate Receptor 2-Lacking AMPA Receptors to Extrasynaptic Plasma Membrane Potentiates Excitotoxicity. J Neurosci 28:2119-2130.
Mac Nair CE, Fernandes KA, Schlamp CL, Libby RT, Nickells RW (2014) Tumor necrosis factor alpha has an early protective effect on retinal ganglion cells after optic nerve crush. J Neuroinflamm 11:194.
Marin I, Kipnis J (2013) Learning and memory … and the immune system. Learning & Memory 20:601-606.
Mayer ML, Westbrook GL (1987) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. J Physiol 394:501-527.
McCord MC, Aizenman E (2014) The role of intracellular zinc release in aging, oxidative stress, and Alzheimer's disease. Front Aging Neurosci 6:77.
Mellor IR (2010) The AMPA receptor as a therapeutic target: current perspectives and emerging possibilities. Future Med Chem 2:877-891.
Mørkve SH, Veruki ML, Hartveit E (2002) Functional characteristics of non-NMDA-type ionotropic glutamate receptor channels in AII amacrine cells in rat retina. J Physiol 542:147-165.
Morrison JC, Johnson EC, Cepurna W, Jia L (2005) Understanding mechanisms of pressure-induced optic nerve damage. Prog Ret Eye Res 24:217-240.
Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC (1997) A rat model of chronic pressure-induced optic nerve damage. Exp Eye Res 64:85 - 96.
Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, et al (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumor-necrosis factor-{alpha}. Nature 385:733-736.
Mueller C, Corazza N, Trachsel-Løseth S, Eugster H-P, Bühler-Jungo M, Brunner T, Imboden MA (1999) Noncleavable transmembrane mouse tumor necrosis factor-α (TNFα)

mediates effects distinct from those of wild-type TNFα in vitro and in vivo. J Biol Chem 274:38112-38118.

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI (2006) Tumor necrosis factor-{alpha} mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J Neurosci 26:12633-12641.

Novrup H, Bracchi-Ricard V, Ellman D, Ricard J, Jain A, Runko E, Lyck L, Yli-Karjanmaa M, Szymkowski D, Pearse D, Lambertsen K, Bethea J (2014) Central but not systemic administration of XPro1595 is therapeutic following moderate spinal cord injury in mice. J Neuroinflamm 11:159.

Ogoshi F, Yin HZ, Kuppumbatti Y, Song B, Amindari S, Weiss JH (2005) Tumor necrosis-factor-alpha (TNF-[alpha]) induces rapid insertion of Ca2+-permeable [alpha]-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate (Ca-A/K) channels in a subset of hippocampal pyramidal neurons. Exp Neurol 193:384-393.

Oikonomou N, Harokopos V, Zalevsky J, Valavanis C, Kotanidou A, Szymkowski DE, Kollias G, Aidinis V (2006) Soluble TNF mediates the transition from pulmonary inflammation to fibrosis. PLoS ONE 1:e108.

Olleros ML, Guler R, Corazza N, Vesin D, Eugster H-P, Marchal G, Chavarot P, Mueller C, Garcia I (2002) Transmembrane TNF induces an efficient cell-mediated immunity and resistance to mycobacterium bovis bacillus Calmette-Guérin infection in the absence of secreted TNF and lymphotoxin-α. J Immunol 168:3394-3401.

Olleros ML, Guler R, Vesin D, Parapanov R, Marchal G, Martinez-Soria E, Corazza N, Pache J-C, Mueller C, Garcia I (2005) Contribution of transmembrane tumor necrosis factor to host defense against mycobacterium bovis bacillus Calmette-Guerin and mycobacterium tuberculosis infections. Am J Pathol 166:1109-1120.

Osswald IK, Galan A, Bowie D (2007) Light triggers expression of philanthotoxin-insensitive Ca2+-permeable AMPA receptors in the developing rat retina. J Physiol 582:95-111.

Park YH, Mueller Ii BH, McGrady NR, Ma H-Y, Yorio T (2015) AMPA receptor desensitization is the determinant of AMPA receptor mediated excitotoxicity in purified retinal ganglion cells. Exp Eye Res 132:136-150.

Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med 184:1397-1411.

Perry VH (1981) Evidence for an amacrine cell system in the ganglion cell layer of the rat retina. Neurosci 6:931-944.

Pribiag H, Stellwagen D (2014) Neuroimmune regulation of homeostatic synaptic plasticity. Neuropharmacology 78:13-22.

Rodriguez AR, de Sevilla Müller LP, Brecha NC (2013) The RNA binding protein RBPMS is a selective marker of ganglion cells in the mammalian retina. J Comp Neurol 522:1411-1443.

Roh M, Zhang Y, Murakami Y, Thanos A, Lee S, Vavvas D, Benowitz L, Miller J (2012) Etanercept, a widely used inhibitor of tumor necrosis factor-α (TNF-α), prevents retinal ganglion cell loss in a rat model of glaucoma. PLoS One 7:e40065.

Sawada H, Fukuchi T, Tanaka T, Abe H (2010) Tumor necrosis factor-α concentrations in the aqueous humor of patients with glaucoma. Invest Ophthalmol Vis Sci 51:903-906.
Schlamp C, Montgomery A, Mac Nair C, Schuart C, Willmer D, Nickells R (2013) Evaluation of the percentage of ganglion cells in the ganglion cell layer of the rodent retina. Mol Vis 19:1387-1396.
Seitz R, Ohlmann A, Tamm E (2013) The role of Müller glia and microglia in glaucoma. Cell and Tissue Research 353:339-345.
Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999) Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc Natl Acad Sci USA 96:2414-2419.
Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003) Modulation of mitochondrial function by endogenous Zn2+ pools. Proc Natl Acad Sci U S A 100:6157-6162.
Sfikakis P (2010) The first decade of biologic TNF antagonists in clinical practice: lessons learned, unresolved issues and future directions. Curr Dir Autoimmun 11:180-210.
Shakoor N, Michalska M, Harris CA, Block JA (2002) Drug-induced systemic lupus erythematosus associated with etanercept therapy. . Lancet 359:579-580.
Singer JH, Diamond JS (2003) Sustained Ca2+ entry elicits transient postsynaptic currents at a retinal ribbon synapse. J Neurosci 23:10923-10933.
Sivakumar V, Foulds WS, Luu CD, Ling E-A, Kaur C (2011) Retinal ganglion cell death is induced by microglia derived pro-inflammatory cytokines in the hypoxic neonatal retina. J Pathol 224:245-260.
Slifman NR, Gershon SK, Lee JH, Edwards ET, Braun MM (2003) Listeria monocytogenes infection as a complication of treatment with tumor necrosis factor alpha-neutralizing agents. Arthritis Rheum 48:319-324.
Soto I, Howell GR (2014) The complex role of neuroinflammation in glaucoma. Cold Spring Harbor Perspectives in Medicine.
Steed PM et al. (2003) Inactivation of TNF Signaling by Rationally Designed Dominant-Negative TNF Variants. Science 301:1895-1898.
Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-[alpha]. Nature 440:1054-1059.
Stellwagen D, Beattie EC, Seo JY, Malenka RC (2005) Differential regulation of AMPA receptor and GABA eceptor trafficking by tumor necrosis factor-{alpha}. J Neurosci 25:3219-3228.
Stromgaard K, Jensen LS, Vogensen SB (2005) Polyamine toxins: development of selective ligands for ionotropic receptors. Toxicon 45:249-254.
Tezel G (2013) Immune regulation toward immunomodulation for neuroprotection in glaucoma. Current Opinion in Pharmacology 13:23-31.
Tezel G, Yang X, Yang J, Wax MB (2004) Role of tumor necrosis factor receptor-1 in the death of retinal ganglion cells following optic nerve crush injury in mice. Brain Res 996:202-212.
Tham Y-C, Li X, Wong TY, Quigley HA, Aung T, Cheng C-Y (2014) Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis. Ophthalmology 121:2081-2090.
van Oosten BW, Barkhof F, Truyen L, Boringa JB, Bertelsmann FW, von Blomberg BM, Woody JN (1996) Increased MRI activity and immune activation in two multiple

sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. Neurology 47:1531-1534.
Veruki ML, Mørkve SH, Hartveit E (2003) Functional properties of spontaneous EPSCs and non-NMDA receptors in rod amacrine (AII) cells in the rat retina. J Physiol 549:759-774.
Vidal-Sanz M, Salinas-Navarro M, Nadal-Nicolás FM, Alarcón-Martínez L, Valiente-Soriano FJ, Miralles de Imperial J, Avilés-Trigueros M, Agudo-Barriuso M, Villegas-Pérez MP (2012) Understanding glaucomatous damage: Anatomical and functional data from ocular hypertensive rodent retinas. Prog Ret Eye Res 31:1-27.
Xin X, Gao L, Wu T, Sun F (2013) Roles of tumor necrosis factor alpha gene polymorphisms, tumor necrosis factor alpha level in aqueous humor, and the risks of open angle glaucoma: a meta-analysis. Mol Vis 19:526-535.
Yang X, Luo C, Cai J, Powell DW, Yu D, Kuehn MH, Tezel G (2011) Neurodegenerative and Inflammatory Pathway Components Linked to TNF-α/TNFR1 Signaling in the Glaucomatous Human Retina. Invest Ophthalmol & Vis Sci 52:8442-8454.
Yin HZ, Hsu C-I, Yu S, Rao SD, Sorkin LS, Weiss JH (2012) TNF-α triggers rapid membrane insertion of Ca2 + permeable AMPA receptors into adult motor neurons and enhances their susceptibility to slow excitotoxic injury. Exp Neurol 238:93-102.
Yuan L, Neufeld AH (2001) Activated microglia in the human glaucomatous optic nerve head. J Neurosci Res 64:523 - 532.
Zalevsky J, Secher T, Ezhevsky SA, Janot L, Steed PM, O'Brien C, Eivazi A, Kung J, Nguyen D-HT, Doberstein SK, Erard F, Ryffel B, Szymkowski DE (2007) Dominant-Negative Inhibitors of Soluble TNF Attenuate Experimental Arthritis without Suppressing Innate Immunity to Infection. J Immunol 179:1872-1883.

Figure 1. TNFα mRNA and protein are rapidly upregulated in experimental glaucoma. (A) Intraocular pressure (IOP) increased gradually after hypertonic saline injection into an episcleral vein. (B, C) Ocular hypertension (OHT) led to progressive loss of RGC soma and axons. (D) RT-PCR analysis of glaucomatous retinas showed rapid TNFα mRNA upregulation after OHT induction compared to intact controls. (E) Densitometry analysis of amplification products with respect ß-actin controls loaded in the same gel, confirmed TNFα gene expression upregulation in glaucoma (n=3, ANOVA *=$p<0.05$). (F) Analysis of protein homogenates showed that retinal TNFα levels increased at 1 and 3 weeks after OHT induction. The lower panel shows the same blot but probed with an antibody that recognizes β-actin to confirm equal protein loading. (G) Densitometry analysis of western blots showing the ratio of TNFα relative to β-actin (n=7, ANOVA, *=$p<0.05$, **=$p<0.01$). (H-M) Immunohistochemistry using antibodies against TNFα and CRALBP, a Müller cell-specific marker, demonstrated that these glial cells upregulate TNFα soon after glaucoma induction. (N-U) Analysis of the inner plexiform layer on whole-mounted retinas revealed that presumptive microglia/macrophages, visualized with Iba1, are a source of TNFα in glaucoma. Scale bars: 50 μm. Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 2. Ocular hypertension increases TNFR1 and TNFR2 expression by RGCs.

(A) Rapid upregulation of TNFR1 and TNFR2 mRNA in glaucomatous retinas was detected by RT-PCR. (B) Densitometry analysis of amplification products with respect to ß-actin control loaded in the same blot confirmed upregulation of TNFR1/2 gene expression (n=4, ANOVA *=$p<0.05$, **=$p<0.01$). Western blot analysis demonstrated increased levels of retinal TNFR1 (C) and TNFR2 (D). The lower panel is the same blot but probed with an antibody against β-actin to confirm equal protein loading. Densitometry showed increased ratio of TNFR1 (E) or TNFR2 (F) proteins relative to β-actin in glaucomatous retinas (n=4, ANOVA, *=$p<0.05$, **=$p<0.01$). (G, H) Retinal immunohistochemistry confirmed an increase in TNFR1- and TNFR2-positive cells. (I, J) Both TNFR1 and TNFR2 were upregulated by RGCs, visualized with the retrograde tracer Fluorogold (FG). Scale bars: 50 μm. INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 3. Inhibition of soluble TNFα promotes RGC soma and axon protection.

(A-C) Flat-mounted retinas from eyes treated with XPro1595 displayed higher densities of DiI-positive RGCs compared to control retinas treated with vehicle at 3 weeks after ocular hypertension (OHT). Scale bars: 50 μm. (D-F) Optic nerves in XPro1595-treated eyes contained more RGC axon fibers with normal morphology compared to control eyes. Scale bars: 10 μm. (G, H) Quantitative analysis confirmed that XPro1595 (black bars) promoted significant RGC soma and axon survival compared to control eyes treated with vehicle (grey bars). The densities of RGC soma and axons in intact, non-glaucomatous Brown Norway rat retinas are shown as reference (white bars, 100% survival). Values are expressed as the mean ± S.E.M. (intact: n=7; XPro1595: n=7, vehicle: n=6, ANOVA, ***=$p<0.001$).

Figure 4. TNFα stimulates the expression of CP-AMPAR by RGCs.

(A) Co^{2+} accumulation in non-injured (intact) retinas, was detected solely in cells of the inner nuclear layer (INL) previously identified as horizontal and AII amacrine cells. (B) Ocular hypertension (OHT) promoted Co^{2+} accumulation in the ganglion cell layer (GCL) and inner plexiform layer (IPL), where RGC soma and dendrites are located (n=6-8/group). Immunohistochemistry with RBPMS, which allowed identification of RGCs in both intact and glaucomatous retinas (C, D), demonstrated Co^{2+} accumulation in RBPMS-positive cells (E-G). Glaucoma-induced Co^{2+} uptake was blocked by XPro1595, GYKI 52466 and PhTx 343 (H-J) indicating that Co^{2+} accumulation in RGCs was due to increased flux of divalent cations exclusively through TNFα-induced CP-AMPAR (n=6-8/group). Scale bars: 20 μm. PS: Photoreceptor Segments; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 5. AMPAR GluA2 subunits are fully edited at the Q/R site in glaucoma.

(A) The presence of an additional *Bbvl* restriction site at the Q/R site was used to determine the editing state of GluA2. *Bbvl* digestion of a 254-bp amplicon comprising the Q/R site led to the appearance of two or three fragments, corresponding to the presence of edited (R) and unedited (Q) GluA2 forms, respectively. (B) The *Bbvl* digestion pattern of the GluA2 amplicon amplified from cDNA generated from intact and glaucomatous retina matched that of the fully edited GluA2 (R) plasmid control. (C-F) Sanger sequencing shows that both uninjured (intact) and glaucomatous (OHT) retinas encoded for arginine (CGG) at the Q/R site (highlighted yellow).

Figure 6. GluA2 is downregulated in RGCs subjected to ocular hypertension.

(A) RT-PCR analysis of glaucomatous retinas showed GluA2 mRNA downregulation after OHT induction compared to intact controls. (B) Densitometry analysis of amplification products with respect to ß-actin controls confirmed GluA2 gene expression downregulation in glaucoma (n=3, ANOVA *=$p<0.05$). (C) Analysis of protein homogenates showed a decrease in retinal GluA2 levels after OHT induction. The lower panel is the same blot probed with an antibody that recognizes β-actin to confirm equal protein loading. (D) Densitometry analysis of western blots, showing the ratio of GluA2 relative to β-actin (n=11, ANOVA, **=$p<0.01$). (E) Subcellular fractionation showed a decrease in membrane-associated GluA2 in hypertensive retinas. Enrichment of each fraction for membrane or cytosolic proteins was confirmed using Na^+/K^+ ATPase or GAPDH, respectively (F) Densitometry analysis revealed a significant decrease of GluA2 in the membrane fraction from ocular hypertensive (OHT) retinas compared to intact controls (n=4, Student's *t* test, *=$p<0.05$). (G, H) Double immunohistochemistry showed that GluA2 (red) co-localized with the RGC-specific marker RBPMS (green), and that GluA2 protein expression decreased in glaucomatous (OHT) retinas. Scale bar: 50 µm. ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 7. CP-AMPAR blockade promotes RGC survival.

(A-C) Flat-mounted retinas from eyes treated with PhTX343 or GYKI 52466 displayed higher densities of DiI-positive RGCs compared to control retinas treated with vehicle at 3 weeks after ocular hypertension (OHT). Scale bars: 50 μm. (D-F) Optic nerves from PhTX343- or GYKI 52466-treated eyes contained many more RGC axon fibers with normal morphology compared to control eyes. Scale bars: 10 μm. (G, H) Quantitative analysis confirmed that PhTX343 (black bars) and GYKI 52466 (dark grey bars) promoted significant RGC soma and axon survival compared to control eyes treated with vehicle (light grey bars). The densities of RGC soma and axons in intact, non-glaucomatous Brown Norway rat retinas are shown as reference (white bars, 100% survival). Values are expressed as the mean ± S.E.M. (intact: n=7; PhTX343: n=6, GYKI 52466: n=6; vehicle: n=6, ANOVA, ***=$p<0.001$, *=$p<0.05$).

II.7. TABLES

Table 1. Intraocular pressure (IOP) elevation in glaucomatous eyes treated with different compounds. Mean and peak IOP elevations for each cohort of rats exposed to 3 weeks of ocular hypertension (OHT) after treatment with XPro1595, GIKY 52466, PhTX 343 or vehicle (n = 5-15/group).

Table 1. Intraocular pressure (IOP) elevation in glaucomatous eyes

Time after OHT surgery	Treatment	N	Mean IOP (mm Hg) ± S.E.M.			Peak IOP (mm Hg)	
			Glaucoma	Control	Difference	Glaucoma	Control
3 weeks	Vehicle	15	36 ± 0.6	24 ± 0.2	12 ± 0.6	39 ± 0.6	26 ± 1.2
	XPro1595	15	37 ± 0.7	25 ± 0.2	12 ± 0.6	41 ± 1.7	26 ± 0.6
	GIKY 52466	7	35 ± 0.8	24 ± 0.5	11 ± 0.7	39 ± 1.4	26 ± 0.5
	PhTx 343	5	35 ± 1.0	24 ± 0.6	11 ± 1.4	39 ± 1.3	26 ± 0.6

II.8. FIGURES

FIGURE 1

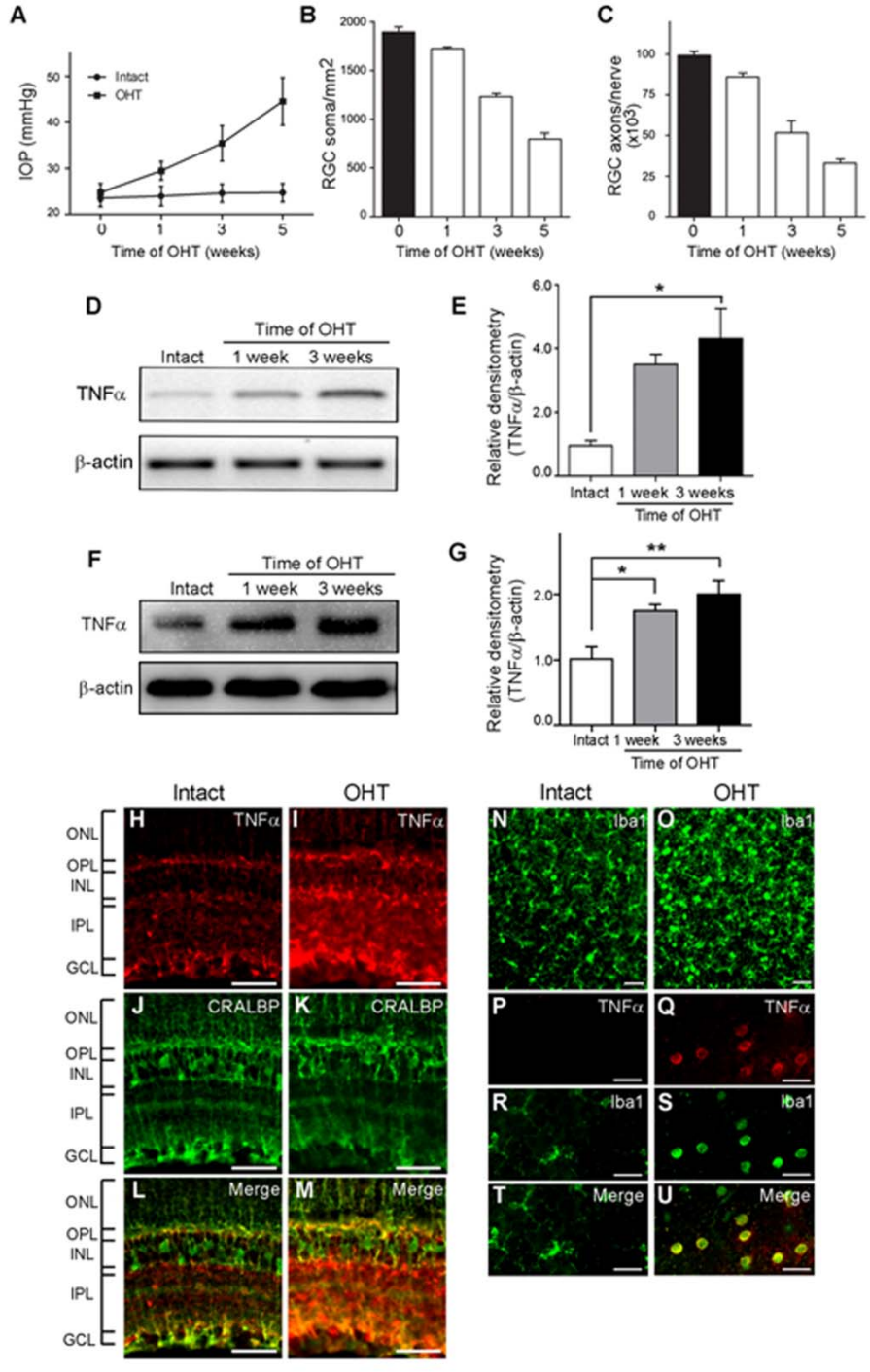


FIGURE 2

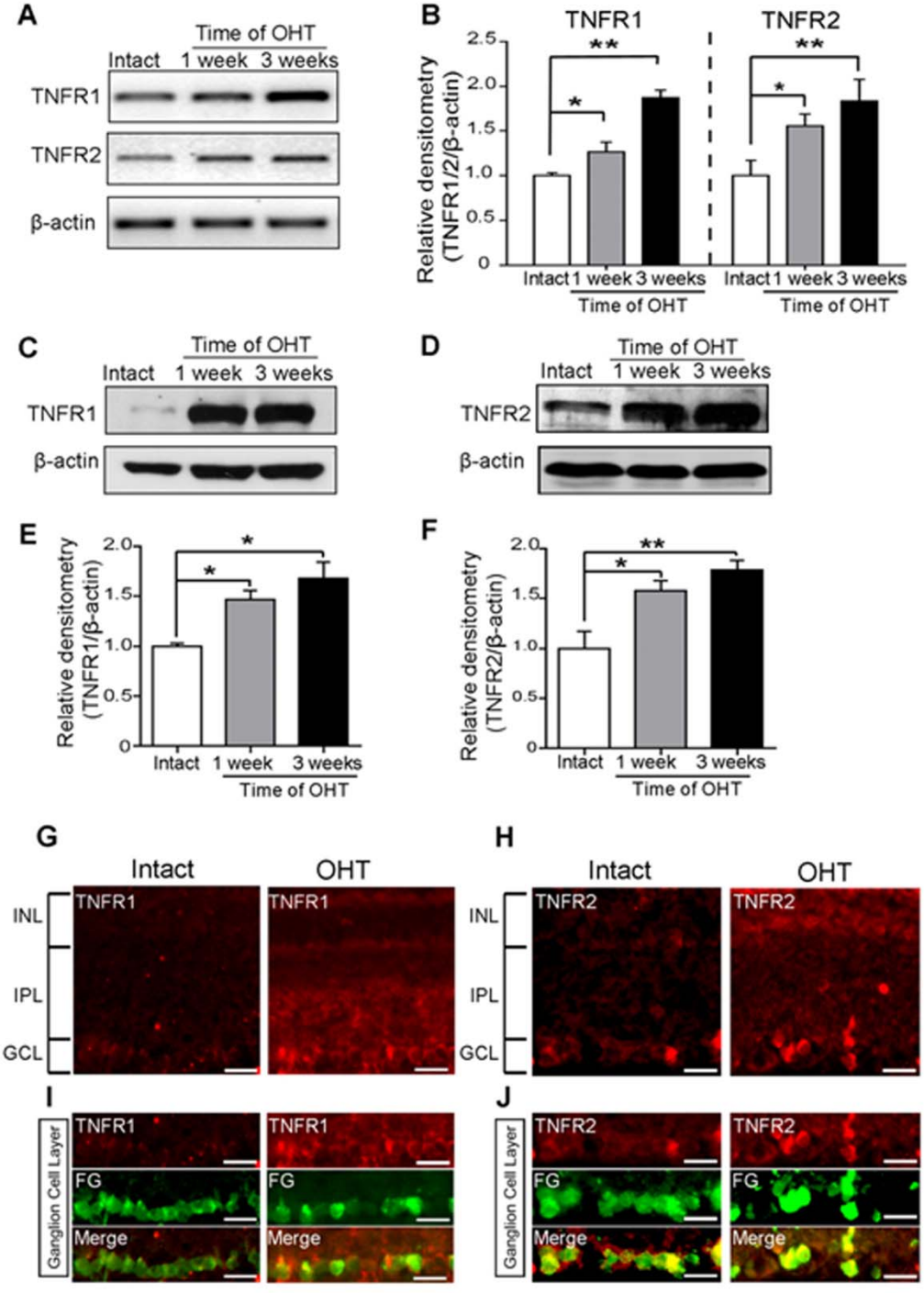

A
Time of OHT
Intact
1 week
3 weeks
TNFR1
TNFR2
β-actin
B
TNFR1
TNFR2
Relative densitometry (TNFR1/2/β-actin)
Intact
1 week
3 weeks
Time of OHT
C
D
E
Relative densitometry (TNFR1/β-actin)
F
Relative densitometry (TNFR2/β-actin)
G
Intact
OHT
INL
IPL
GCL
H
I
J
Ganglion Cell Layer
FG
Merge

FIGURE 3

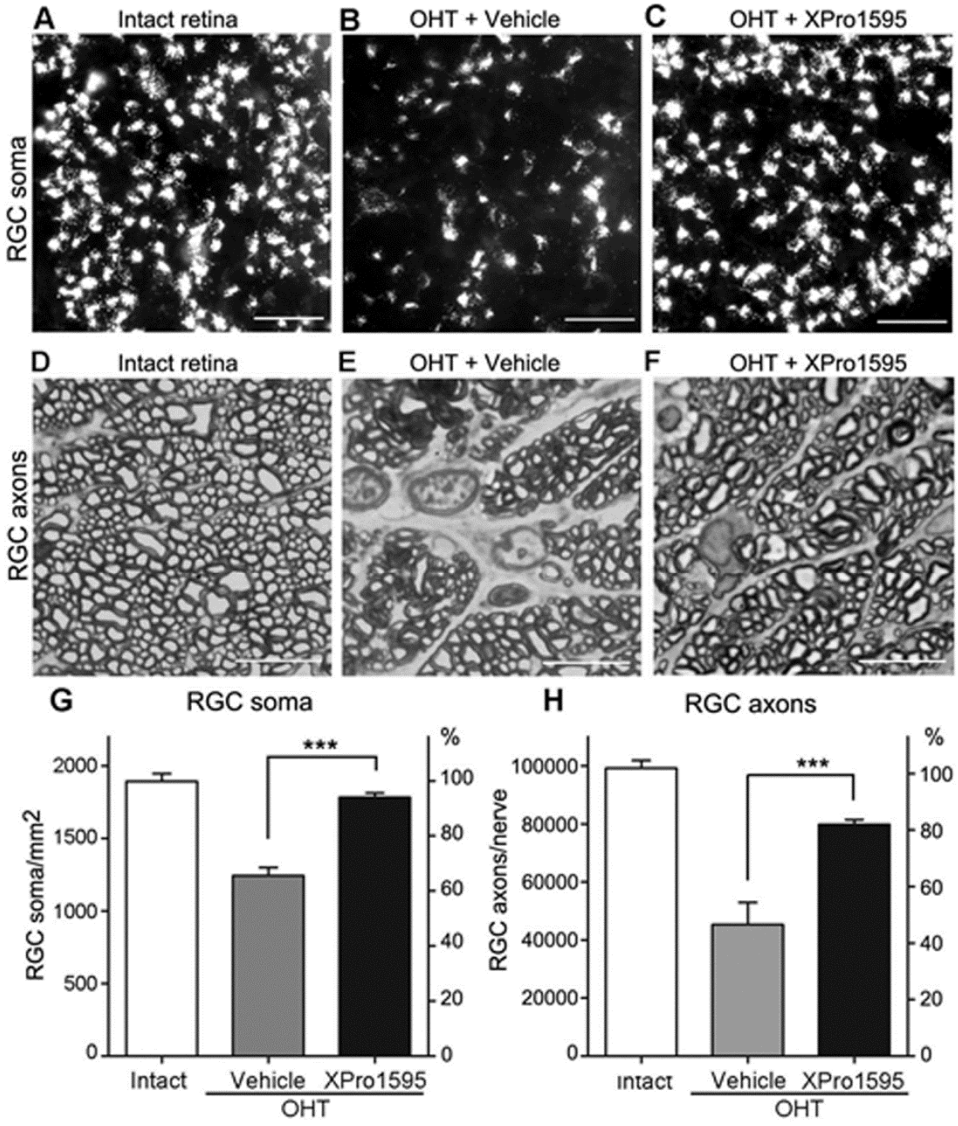

A
Intact retina
B
OHT + Vehicle
C
OHT + XPro1595
RGC soma
D
Intact retina
E
OHT + Vehicle
F
OHT + XPro1595
RGC axons
G
RGC soma
RGC soma/mm2
2000
1500
1000
500
0
%
100
80
60
40
20
0
***
Intact
Vehicle
XPro1595
OHT
H
RGC axons
RGC axons/nerve
100000
80000
60000
40000
20000
0
%
100
80
60
40
20
0
***
Intact
Vehicle
XPro1595
OHT

FIGURE 4

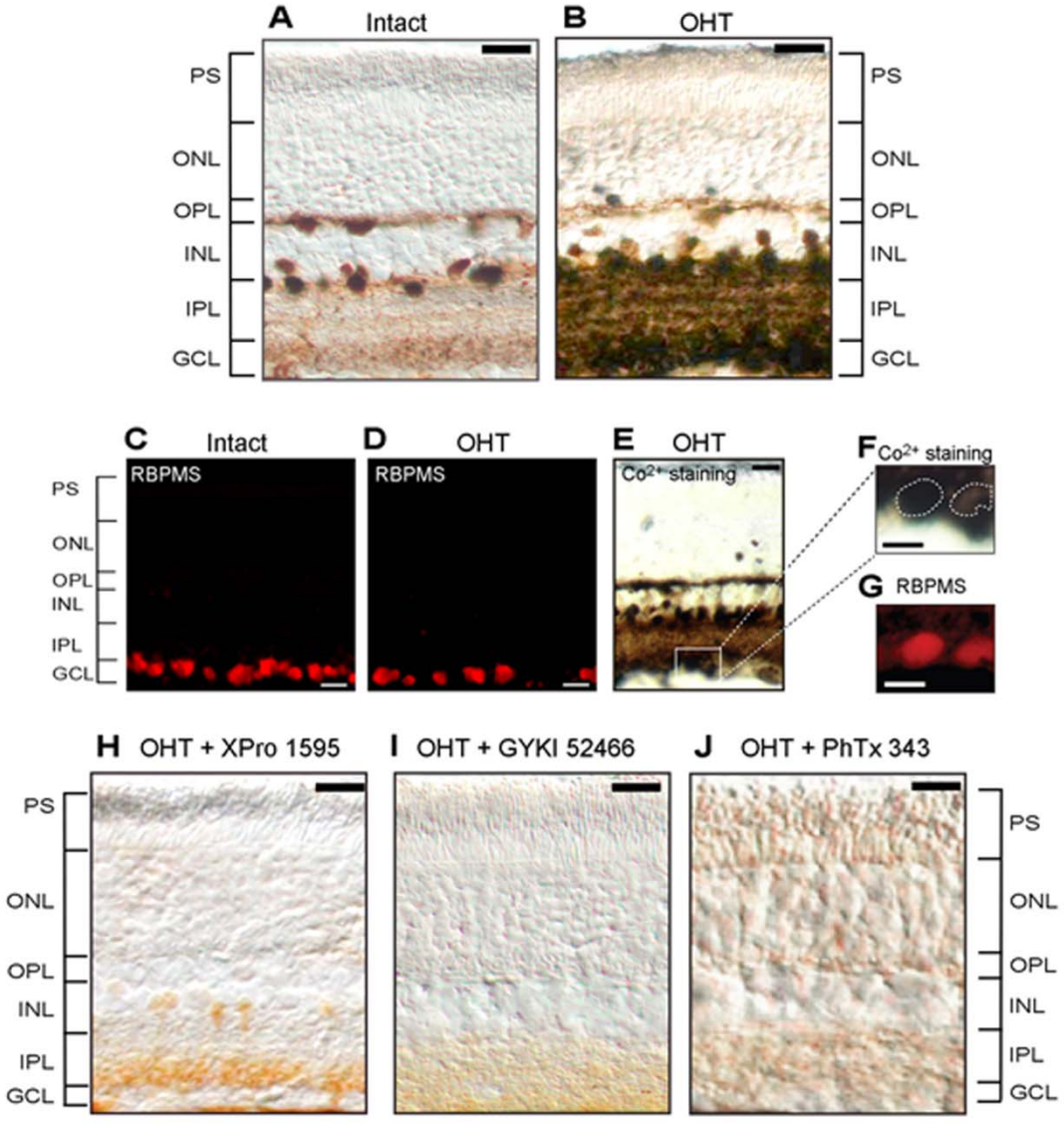

A
Intact
B
OHT
PS
ONL
OPL
INL
IPL
GCL
C
Intact
D
OHT
E
OHT
F
Co2+ staining
RBPMS
Co2+ staining
G
RBPMS
H
OHT + XPro 1595
I
OHT + GYKI 52466
J
OHT + PhTx 343

FIGURE 5

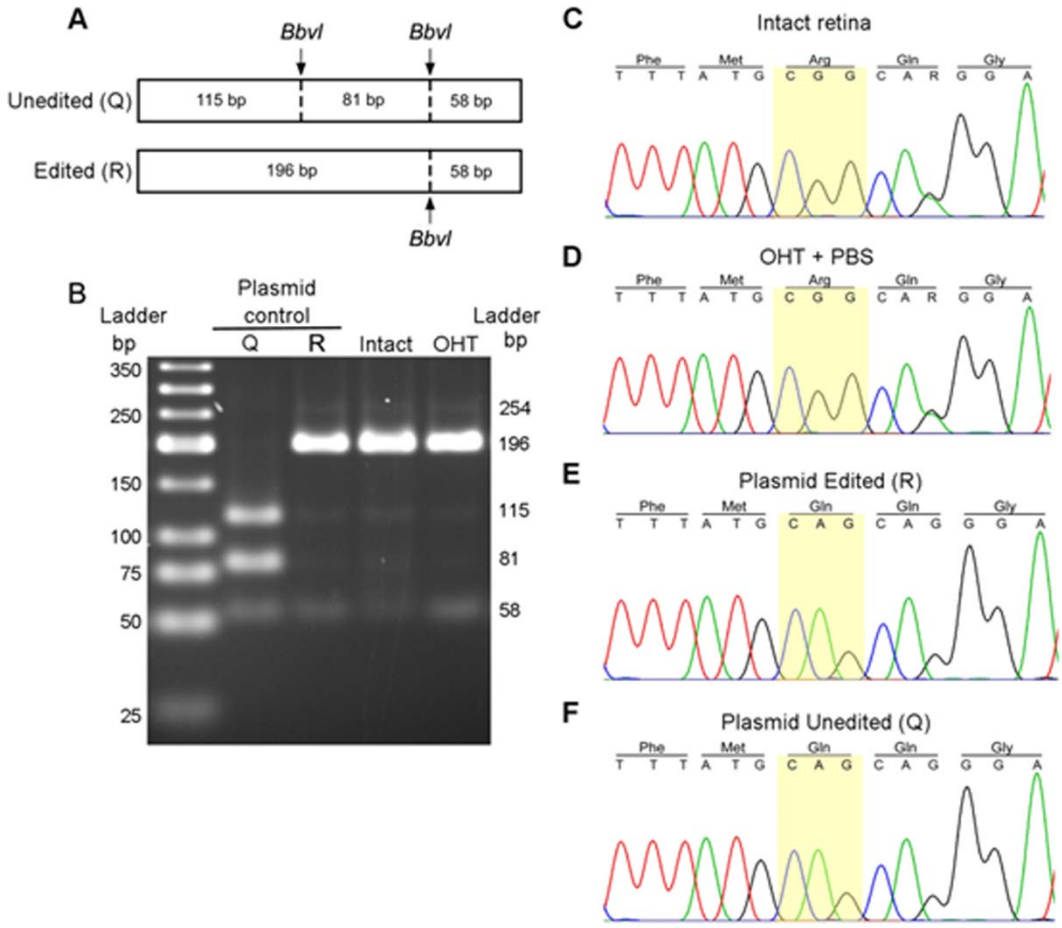

A
BbvI
BbvI
Unedited (Q)
115 bp
81 bp
58 bp
Edited (R)
196 bp
58 bp
BbvI
B
Ladder bp
Plasmid control
Q
R
Intact
OHT
Ladder bp
350
250
150
100
75
50
25
254
196
115
81
58
C
Intact retina
D
OHT + PBS
E
Plasmid Edited (R)
F
Plasmid Unedited (Q)
Phe
Met
Arg
Gln
Gly

FIGURE 6

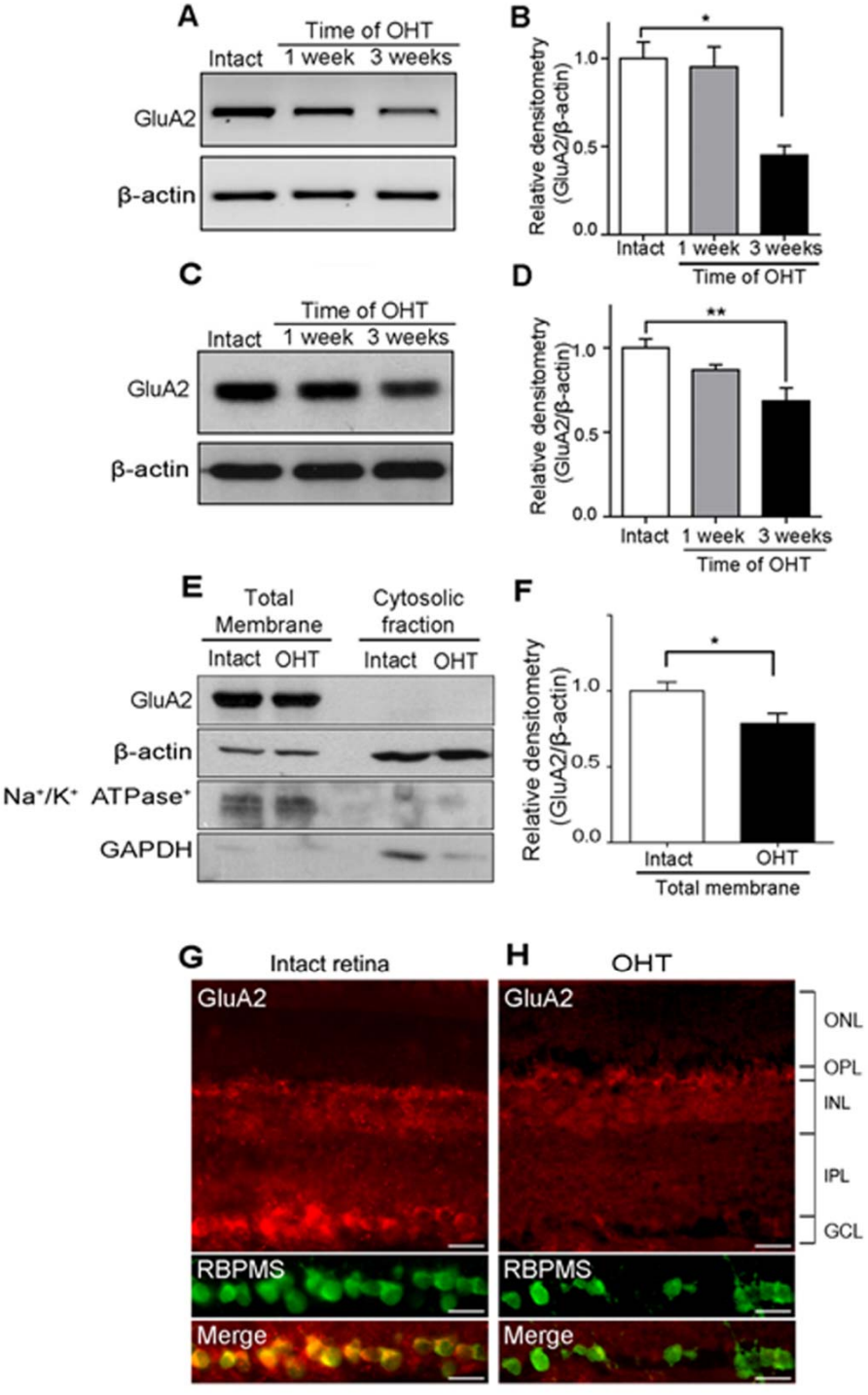
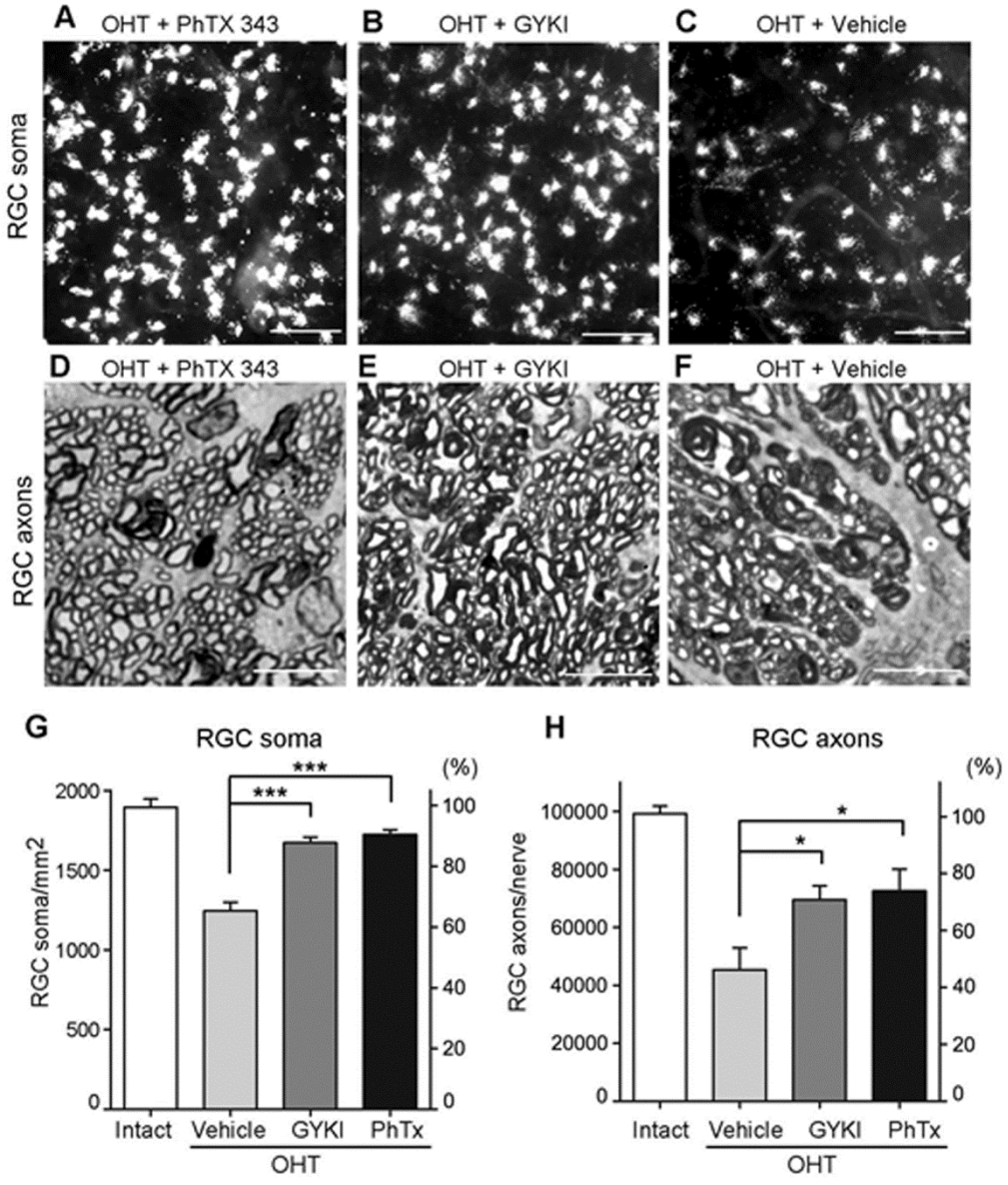

A
Time of OHT
Intact
1 week
3 weeks
GluA2
β-actin
B
Relative densitometry (GluA2/β-actin)
1.0
0.5
0.0
*
Intact
1 week
3 weeks
Time of OHT
C
Time of OHT
Intact
1 week
3 weeks
GluA2
β-actin
D
Relative densitometry (GluA2/β-actin)
1.0
0.5
0.0
**
Intact
1 week
3 weeks
Time of OHT
E
Total Membrane
Cytosolic fraction
Intact
OHT
Intact
OHT
GluA2
β-actin
Na+/K+ ATPase+
GAPDH
F
Relative densitometry (GluA2/β-actin)
1.0
0.5
0.0
*
Intact
OHT
Total membrane
G
Intact retina
H
OHT
GluA2
GluA2
ONL
OPL
INL
IPL
GCL
RBPMS
RBPMS
Merge
Merge

FIGURE 7


A
OHT + PhTX 343
B
OHT + GYKI
C
OHT + Vehicle
RGC soma
D
OHT + PhTX 343
E
OHT + GYKI
F
OHT + Vehicle
RGC axons
G
RGC soma
RGC soma/mm2
2000
1500
1000
500
0
(%)
100
80
60
40
20
0
***
***
Intact
Vehicle
GYKI
PhTx
OHT
H
RGC axons
RGC axons/nerve
100000
80000
60000
40000
20000
0
(%)
100
80
60
40
20
0
*
*
Intact
Vehicle
GYKI
PhTx
OHT

CHAPTER 3

III. SECOND ARTICLE: THE GLIAL CELL MODULATOR IBUDILAST ATTENUATES THE NEUROINFLAMMATORY RESPONSE AND ENHANCES VIABILITY IN GLAUCOMA"

To be submitted.

The glial cell modulator ibudilast attenuates the neuroinflammatory response and enhances neuronal viability in glaucoma.

Abbreviated title: Ibudilast and gliosis-induced retinal neuron death.

Jorge L. Cueva-Vargas [1*], Nicolas Belforte [1*], and Adriana Di Polo [1].

[1] Department of Neuroscience and University of Montreal Hospital Research Center (CRCHUM), University of Montreal, Montreal, QC H2X 0A9, Canada

Corresponding author: Adriana Di Polo, Ph.D.
Dept. of Neuroscience
University of Montreal
CRCHUM, 900 Rue Saint-Denis
Tour Viger, Room R09.720
Montreal, Quebec H2X 0A9, Canada

[*] These author contributed equally to this study.

Number of pages: 32
Number of figures and tables: Figures (8), Tables (1)
Number of words: Abstract (260), Introduction (804), Discussion (1666)
Conflict of Interest: The authors declare no competing financial interests.

Acknowledgements: This work was supported by a grant from the Canadian Institutes of Health Research (A.D.P.). N.B. is the recipient of a postdoctoral fellowship from the Fonds de recherche du Québec-Santé (FRQS) and a H.H. Jasper scholarship from the Groupe de Recherche sur le Système Nerveux Central (GRSNC). A.D.P. is a Chercheur Boursier National FRQS.

III.1. ABSTRACT

Glaucoma is the leading cause of irreversible blindness worldwide. Loss of vision in glaucoma results from the loss of retinal ganglion cells (RGCs). Glial cell-mediated neuroinflammation has been proposed to contribute to glaucoma pathophysiology, but whether this response is damaging or beneficial for RGC survival is not well understood. To test this, we characterized the role of ibudilast, a clinically approved glial cell modulator and a cAMP phosphodiesterase (PDE) inhibitor with preferential affinity for PDE type 4 (PDE4). Using a rat model of ocular hypertension, our data demonstrate that intraocular administration of ibudilast effectively attenuated macroglia and microglia reactivity in the retina and optic nerve. Reduced glial cell activation correlated with decreased production of proinflammatory cytokines including tumor necrosis factor α, interleukin-1β, interleukin-6 and macrophage migration inhibitory factor. Importantly, ibudilast treatment promoted robust RGC soma and axonal survival in glaucomatous eyes. Immunohistochemical analysis of intact retinas revealed abundant PDE4 expression in neurons of the inner nuclear and ganglion cell layers. Intriguingly, ocular hypertension triggered substantial PDE4A expression in Müller glia, which correlated with accumulation of cAMP in these cells after ibudilast treatment. Co-administration of ibudilast with Rp-cAMPS, a cell-permeable and non-hydrolysable cAMP analog that inhibits protein kinase A (PKA), completely obliterated the neuroprotective effect of ibudilast. Our study demonstrates that ibudilast, a safe and well-tolerated glial cell modulator, attenuates gliosis and production of proinflammatory mediators in glaucoma, and enhances neuronal viability through activation of the cAMP/PKA pathway. Collectively, these findings identify PDE4/cAMP/PKA signaling as a potential target for the modulation of the harmful effects associated with chronic neuroinflammation in glaucoma.

III.2. INTRODUCTION

Glaucoma is an optic neuropathy and a leading cause of irreversible blindness that affects over 60 million people worldwide (Tham et al. 2014). Vision loss in glaucoma results from the progressive and relentless loss of retinal ganglion cells (RGCs) and their axons in the optic nerve. High intraocular pressure (IOP) is a major risk factor for developing glaucoma and, as such, therapies have focused on pharmacological or surgical strategies to lower eye pressure (Weinreb and Khaw 2004). In many cases, however, visual deficits continue to progress in spite of effective pressure control, indicating that mechanisms other than elevated IOP contribute to disease progression (Schwartz and London 2008).

Accumulating evidence suggests that neuroinflammation plays a role in RGC death in glaucoma (Soto and Howell 2014a; Tezel 2013). Indeed, a number of studies have confirmed the presence of hallmark features of neuroinflammation in glaucoma animal models as well as in human specimens including glial cell activation (Bosco et al. 2011; Ebneter et al. 2010; Hernandez et al. 2008; Inman and Horner 2007; Johnson and Morrison 2009; Sun et al. 2013; Wang et al. 2002; Yuan and Neufeld 2001), increased proinflammatory cytokine signaling (Howell et al. 2012; Johnson et al. 2011; Tezel et al. 2012; Yang et al. 2011a), induction of the complement cascade (Ahmed et al. 2004; Howell et al. 2011; Kuehn et al. 2008; Ren and Danias 2010; Stasi et al. 2006; Stevens et al. 2007; Tezel et al. 2010), and trans-endothelial migration of leukocytes (Howell et al. 2012). Although chronic inflammation can be damaging for neurons, it has been recognized that the recruitment of immune cells is an integral component of the central nervous system (CNS) ability for self-repair and for the resolution of inflammation (Schwartz and Baruch 2014; Schwartz and London 2009). Based on this, we put forward the hypothesis that

strategies that attenuate the inflammatory response in glaucoma, but do not completely eliminate it, might have a beneficial effect on neuronal viablity.

To test this, we characterized the role of ibudilast (3-isobutyryl-2-isopropylpyrazolo[1,5-a]pyridine), a non-selective 3',5'-cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE) inhibitor with preferential affinity for PDE type 4 (PDE4) (Gibson et al. 2006; Huang et al. 2006). Ibudilast is a glial cell modulator that attenuates gliosis and decreases the production of proinflammatory cytokines (Mizuno et al. 2004; Rodgers et al. 2014; Suzumura et al. 1999). Ibudilast has been clinically used for almost two decades for the treatment of asthma and post-stroke dizziness, and is currently being developed as a potential therapy for multiple sclerosis, neuropathic pain and drug addiction (Rolan et al. 2009).

Using a rat model of ocular hypertension, we demonstrate that ibudilast effectively attenuated glial cell activation in the retina and optic nerve, and reduced the levels of proinflammatory cytokines. Importantly, ibudilast promoted robust survival of RGC soma and axons exposed to ocular hypertension. Of interest, elevated IOP triggered PDE4A upregulation in Müller glia, and ibudilast treatment resulted in cAMP accumulation in these cells. Co-administration of ibudilast with Rp-cAMPS, an inhibitor of the cAMP-dependent protein kinase (PKA), blocked ibudilast-mediated neuroprotection. Collectively, our findings demonstrate that the glial cell modulator ibudilast promotes substantial RGC soma and axon survival in a PKA-dependent manner, and suggest that ibudilast might counter the injurious phenotype associated with neurodegeneration, by increasing cAMP levels in Müller cells.

III.3. MATERIALS AND METHODS

III.3.1. Experimental animals

All procedures were performed in male Brown Norway rats (300–400 g; Charles River Laboratories International, Inc., St-Constant, QC, Canada) in compliance with the guidelines of the Canadian Council on Animal Care for the Use of Experimental Animals (http://www.ccac.ca). The number of animals used in each experiment is indicated in the corresponding figure legend and table.

III.3.2. Ocular hypertension

Unilateral elevation of intraocular pressure was induced by injection of hypertonic saline solution into an episcleral vein as previously described (Morrison model) (Cueva Vargas et al. 2015; Morrison et al. 1997). Briefly, a plastic ring was applied to the ocular equator to confine the injection to the limbal plexus and a microneedle was used to inject 50 µl of sterile 1.85 M NaCl through an episcleral vein. Following injection, the plastic ring was removed and the eyes were examined to assess the extent to which the saline solution traversed the limbal microvasculature. Polysporin ophthalmic ointment (Pfizer Canada Inc., Kirkland, QC) was applied to the operated eye and the animal was allowed to recover. Eye pressure measurements were taken from awake animals after corneal application of one drop of proparacaine hydrochloride (0.5%, Alcon Laboratories, Inc., Fort Worth, TX) using a calibrated tonometer (TonoPen XL; Medtronic Solan, Jacksonville, FL). The tonometer was held perpendicular to the corneal surface and ten consecutive readings per eye were taken and averaged. Intraocular pressure was measured every other day, at the same time, for the entire duration of the experiment. The mean and peak (maximum) intraocular pressure values for each eye were calculated and used to estimate the mean and peak pressure for experimental and control groups.

III.3.3. Drug delivery

The following compounds were injected at one and two weeks after OHT induction into the vitreous chamber in a total volume of 5 µl using a Hamilton syringe adapted with a 32-gauge glass microneedle: Ibudilast (3-isobutyryl-2-isopropylpyrazolo[1,5-a] pyridine, 500 µM, Sigma, St Louis, MO), Rp-cAMPS (Rp-Adenosine 3',5'-cyclic monophosphorothioatetriethylammonium salt, 500 µM; Sigma), or rolipram (150 µM; Sigma). Control animals received sterile phosphate buffer (PBS) vehicle. The tip of the needle was inserted into the superior hemisphere of the eye at a ~45° angle through the sclera into the vitreous body to avoid retinal detachment or injury to eye structures. Surgical glue (Indermill, Tyco Health Care, Mansfield, MA) was used to seal the injection site. Animals were sacrificed three weeks after OHT induction.

III.3.4. Retinal immunohistochemistry

Flat mounted retinas: Rats were perfused transcardially with 4% paraformaldehyde (PFA), the eyes were immediately collected, and the retinas were carefully dissected out. Retinas were free floated overnight at 4°C in blocking solution: 10% normal goat serum (NGS) or normal donkey serum (NDS), 2.5% BSA, and 0.5% Triton X-100 in PBS. Retinas were then incubated for 72 h a 4°C in blocking solution containing an antibodies against: brain-specific homeobox/POU domain protein 3 (Brn3a; 0.3 µg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), cluster of differentiation 68 (CD68, 5 µg/ml, Millipore, Temeluca, CA), anti-ionized calcium binding adapter molecule-1 (Iba1, 1.5 µg/ml, Wako Chemicals, Osaka, JA), or TNFα (4 µg/ml, R&D Systems, Minneapolis, MN). Samples were then incubated in secondary donkey anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG Alexa Fluor 488 (6 µg/ml Molecular Probes, Eugene, OR). The retinas were washed and flat-mounted on glass slides with the retinal ganglion

cell layer side up for visualization using a fluorescent microscope (Zeiss AxioSkop 2 Plus, Carl Zeiss, Canada).

Retinal cross sections: Animals were perfused transcardially with 4% PFA and retinal cryosections (16 µm) were prepared. The following primary antibodies were added to the retinal sections in blocking solution and incubated overnight at 4°C: Glial fibrillary acidic protein (GFAP, 1 µg/ml, Millipore), CD68 (2 µg/ml, Millipore), cellular retinaldehyde-binding protein (CRALBP, 1 µg/ml; ThermoFisher Scientific, Waltham, MA), Iba1 (1.5 µg/ml, Wako Chemicals), RNA-Binding Protein with Multiple Splicing (RBPMS, 0.01 µg/ml, PhosphoSolutions, Aurora, CO), glutamine synthetase (GS, Sigma), phosphodiesterase 4 types A, B and D (PDE4A, B and D; 2.5 µg/ml, Abcam, Cambridge, MA), or cAMP (1 µg/ml, Abcam). Sections were washed and incubated with secondary antibodies: anti-rabbit IgG (1 µg/ml, Cy3, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or anti-mouse IgG (1 µg/ml, FITC, Sigma).

III.3.5. RGC and microglia quantification

RGC soma counts: Whole retinas labeled with Brn3a were washed and flat-mounted on glass slides with the retinal ganglion cell layer side up for visualization using a fluorescent microscope (Zeiss AxioSkop 2 Plus, Carl Zeiss, Canada). Brn3a-labeled RGCs were counted in three square areas at 1, 2 and 3 mm from the optic disc in each retinal quadrant for a total of 12 retinal areas encompassing a total area of 1 mm^2.

RGC axon counts: Animals received a transcardial injection of heparin (1000 u/kg) and sodium nitroprusside (10 mg/kg) followed by perfusion with 2% PFA and 2.5% glutaraldehyde in 0.1 M PBS. Optic nerves were dissected, fixed in 2% osmium tetroxide, and embedded in epon resin. Semi-thin sections (0.7 µm) were cut on a microtome (Reichert, Vienna, Austria) and

stained with 1% toluidine blue. RGC axons were counted at 1 mm from the optic nerve head in five non-overlapping areas of each optic nerve section, encompassing a total area of 5,500 μm^2 per nerve. The five optic nerve areas analyzed included: one in the center of the nerve, two peripheral dorsal and two peripheral ventral regions. The total area per optic nerve cross-section was measured using Northern Eclipse image analysis software (Empix Imaging, Toronto, ON), and this value was used to estimate the total number of axons per optic nerve.

Microglia counts: Whole retinas labeled with CD68 were washed and flat-mounted on glass slides with the retinal ganglion cell layer side up. CD68-positive cells were counted in three square areas at 1, 2 and 3 mm from the optic disc in each retinal quadrant for a total of 12 retinal areas encompassing a total area of 1 mm^2. CD68-positive cells were also quantified in 3 non-overlapping square areas (0.077 mm^2/each) on longitudinal sections of optic nerve.

III.3.6. Western blot analyses

Retinal samples were homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM Na fluoride, 0.25% Na deoxycholate and 2 nM $NaVO_3$ supplemented with protease and phosphatase inhibitors. Samples were separated in 7.5-15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Blots were incubated overnight at 4°C in blocking buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1 % Tween-20 and 5% bovine serum albumin) containing each of the following primary antibodies: TNFα, interleukin 1β (IL-1β), interleukin 6 (IL-6) (1 μg/ml, R&D Systems), migratory macrophage inhibitory factor (MIF, 1 μg/ml, Abcam), or β-actin (0.5 μg/ml; Sigma). Membranes were washed and incubated in peroxidase-linked anti-mouse or anti-rabbit secondary antibodies (0.5 μg/ml, GE Healthcare, Mississauga, ON). Blots were developed using a chemiluminescence

reagent (Bio-Rad, Mississauga, ON) followed by exposure of blots to ChemiDoc MP System (Bio-Rad). Densitometric analysis was performed using Image Lab capture and analysis software (Bio-Rad) on scanned autoradiographic films obtained from a series of three independent western blots each carried out using retinal samples from distinct experimental or control groups.

III.3.7. Statistical analyses

Data analysis and statistics were performed using the GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA), followed by the Tukey's Multiple Comparison *post hoc* test, or by a Student's *t* test as indicated in the legends.

III.4. RESULTS

III.4.1. Ibudilast attenuates astrocyte reactivity in experimental glaucoma.

Astrocyte activation is a hallmark of retinal and optic nerve injury (Hernandez et al 2008). To investigate the effect of ibudilast on astrocytic changes in experimental glaucoma, retinal cross sections were evaluated using an antibody against glial fibrillary acidic protein (GFAP). A basal level of GFAP labeling was detected in astrocytic processes in the retinal ganglion cell layer, as well as astrocytes in the optic nerve head and optic nerve proper of the intact (non-injured) eyes (Fig. 1A, D, G). Three weeks after induction of ocular hypertension, a marked increase in GFAP immunoreactivity was observed in retinal astrocytes as well as the proximal Müller cell radial processes (Fig. 1 B). Similarly, GFAP labeling was noticeably upregulated in astrocytes residing in the optic nerve (Fig. 1E, H). Intraocular administration of ibudilast resulted in a significant attenuation of GFAP immunostaining both in the retina and optic nerve of glaucomatous eyes (Fig. 1C, F, I). Western blot analysis of fresh retinal

homogenates demonstrated a robust increase in GFAP protein levels in retinas exposed to ocular hypertension compared to intact controls (Fig. 1J, K). Ibudilast treatment resulted in a substantial decrease of retinal GFAP expression in glaucoma (Fig. 1J, K), consistent with reduced astrogliosis. We conclude that ibudilast effectively attenuates astrocyte and Müller cell reactivity in glaucomatous eyes.

III.4.2. Proliferation and activation of microglia/macrophages are reduced by ibudilast.

RGC death has been shown to correlate with microglia activation and proliferation (Rojas et al. 2014; Wohl et al. 2010). To determine whether ibudilast exerted an effect on microgliosis, we first carried out retinal and optic nerve immunolabeling with Iba1, a pan-microglia and macrophage marker (Imai and Kohsaka 2002). Ocular hypertension resulted in a marked increase in the number of macroglia/macrophages detected in retinal cross section using Iba1 (Fig. 2A, B). On flat-mounted retinas, analysis of the inner plexiform layer revealed reduced ramifications in Iba1-positive cells, suggestive of an activated state, during ocular hypertension (Fig. 2D, E). Ibudilast treatment effectively decreased the number of Iba1-positive cells (Fig. 2C, F). Staining of flat-mounted retinas with Iba1 and CD68, a marker of active microglia/macrophages with a phagocytic phenotype (Sanchez-Guajardo et al. 2010; Travaglione et al. 2002), demonstrated a substantial increase in the number of double-labeled cells, which was markedly reduced in the presence of ibudilast (Fig. 2G-O). Quantitative analysis confirmed that ocular hypertension resulted in a 5.3-fold increase in the number of CD68/Iba1-positive cells compared to intact retinas, and that ibudilast treatment significantly attenuated this response (Fig. 2 P).

Analysis of the optic nerve at the levels of its head and proximal segment also revealed a striking increase in the number of Iba1-positive cells, which was effectively reduced with ibudilast treatment (Fig. 3A-F). Double labeling with Iba1 and CD68 revealed a marked increase

in the number of active microglia/macrophages in glaucomatous eyes, which was reduced by ibudilast (Fig. 3 G-O). Similar to the retina, quantitative analysis confirmed a 2.4-fold increase in CD68/Iba1-positive cells in optic nerves subjected to ocular hypertension, and a tangible decrease in the number of active microglia/macrophages with ibudilast (Fig. 3P). We conclude that there is important microgliosis following induction of ocular hypertension, characterized by activation and increased density of microglia/macrophages, and that ibudilast can effectively reduce this response.

III.4.3. Levels of pro-inflammatory cytokines are attenuated by ibudilast in experimental glaucoma.

Glial cell reactivity has been associated with increased production of pro-inflammatory cytokines (Soto and Howell 2014b). To assess whether ibudilast influenced the global level of cytokines, we performed western blot analyses of retinal homogenates from glaucomatous and control eyes. Our data demonstrate that TNFα is upregulated in glaucomatous retinas at three weeks after induction of ocular hypertension, and that intravitreal administration of ibudilast significantly reduced the levels of retinal TNFα to those found in uninjured retinas (Fig 4A). The efficacy of ibudilast to modulate the expression of other proinflammatory cytokines including IL-1β, IL-6 and MIF was also evaluated. Similar to TNFα, western blot analysis showed that IL-β, IL-6 and MIF were upregulated in glaucoma and that ibudilast treatment decrease the amount of these cytokines to basal levels found in intact retinas (Fig 4 C, E, F). These findings were confirmed by densitometric analysis (Fig. 4C-D, G-H). We conclude that intravitreal injection of ibudilast markedly attenuates the expression of TNFα, IL-1β, IL-6 and MIF in experimental glaucoma.

III.4.4. Ibudilast protects RGC soma and axon protection from ocular hypertension damage.

We next sought to determine whether ibudilast protected RGCs from ocular hypertension-induced damage. Ibudilast was injected intraocularly at 1 and 2 weeks after induction of ocular hypertension, and RGC survival was evaluated a week later (3 weeks after injury onset). Cell soma and axon quantification was performed blinded to treatment. Flat-mounted retinas from eyes treated with ibudilast showed higher densities of cells positive for Brn3a, an RGC-specific marker (Nadal-Nicolas et al. 2009), compared to control retinas treated with vehicle (PBS) (Fig. 5A-C). Quantitative analysis demonstrated that ibudilast promoted significant RGC survival (92%: 1884 ± 44 RGCs/mm^2, mean ± S.E.M., n=5) with respect to eyes that received vehicle (68%: 1399 ± 128 RGCs/mm^2, n=6) (Fig. 5G).

A hallmark of glaucoma is the degeneration of RGC axons in the optic nerve posterior to the lamina cribrosa, therefore, we also investigated the capacity of ibudilast to protect axons. Analysis of optic nerve cross-sections showed a substantially larger number of RGC axon fibers with normal morphology in ibudilast-treated eyes compared to vehicle-treated controls (Fig. 5D-F). The latter displayed extensive axon degeneration including disarray of fascicular organization and degradation of myelin sheaths. Quantitative analysis confirmed that ibudilast promoted marked protection of RGC axons (91%: 90640 ± 2658 axons/nerve, mean ± S.E.M., n=5) compared to vehicle treatment (61%: 61319 ± 8112 axons/nerve, n=6) (Fig. 5H). To assess whether ibudilast reduced intraocular pressure, which could account for its neuroprotective effect, we measured eye pressure every other day after glaucoma induction. Our results show that ibudilast administration did not reduce eye pressure (Table 1). Given that the rate of RGC death is proportional to ocular hypertension, the similar increase in intraocular pressure amongst

groups allowed for reliable comparison of the neuroprotective effect of ibudilast versus vehicle. Our results demonstrate that ibudilast protects both RGC soma and axons, without altering eye pressure.

III.4.5. Ocular hypertension triggers PDE4A expression in Müller cells.

Next we sought to identify the cellular targets of ibudilast action in the retina. Ibudilast exerts its function by inhibiting cAMP PDE activity and has a demonstrated preferential affinity for PDE4 (Huang et al. 2006). In mammals, there are multiple variants of PDE4 encoded by four non-redundant genes (PDE4A-D) (Houslay et al. 2005). In the rat retina, only PDE4A, PDE4B and PDE4D are expressed, with PDE4A being the most abundant (Whitaker and Cooper 2009). Immunohistochemical analysis confirmed that PDE4A is expressed in many cells of the inner nuclear and ganglion cell layers in the intact, non-injured retina (Fig. 6A). Similar to previous studies, we found that PDE4A was expressed by RGCs, visualized with the RGC-specific marker RNA-Binding Protein with Multiple Splicing (RBPMS) (Fig. 6B-D). In glaucomatous retinas, however, in addition to PDE4A-positive RGCs, we detected the appearance of strongly labeled radial processes resembling presumptive Müller glia (Fig. 6E). Co-localization of PDE4A with the Müller cell-specific marker Cellular Retinaldehyde Binding Protein (CRALBP) confirmed that ocular hypertension triggered PDE4A expression in these glial cells (Fig. 6F-H). In contrast, we did not detect PDE4A expression in Iba1-positive microglia/macrophages in glaucomatous retinas (Fig. 6I-K). PDE4B and PDE4D were also detected in cells of the inner nuclear and ganglion cell layers, including RGCs, but their expression pattern did not change with glaucoma (Fig. 6L-S).

To establish whether ibudilast altered cAMP levels in the retina, we performed immunohistochemical analysis using an antibody against cAMP (Fig. 7). Our results show that

intraocular injection of ibudilast stimulated a marked increase in cAMP in glaucomatous retinas compared to vehicle-treated controls (Fig. 7A-C), consistent with its activity as a cAMP PDE inhibitor. Interestingly, robust accumulation of cAMP was observed in Müller cell end-feet and radial processes, visualized with the specific marker glutamine synthetase (GS) (Fig 7C-E). In addition, cAMP was also observed in RGCs, identified with RBPMS (Fig. 7F-H). We conclude that ibudilast results in cAMP accumulation in target cells, most notably in Müller glia.

III.4.6. The neuroprotective effect of ibudilast is mediated by protein kinase A (PKA) activation.

cAMP exerts its biological functions primarily by activating PKA (Scott 1991). Therefore, we asked whether the effect of ibudilast on the glial response and subsequent neuroprotection in glaucoma involved PKA activation. To test this, we administered ibudilast in combination with Rp-cAMPS, a cell permeable, non-hydrolysable cAMP analog and a competitive inhibitor of PKA (Dostmann 1995). Our data show that co-administration of ibudilast and Rp-cAMPS completely blocked the pro-survival effect of ibudilast on RGC soma and axons to levels found in vehicle-treated eyes (Fig. 8A, B; light grey bars). Injection of Rp-cAMPS into intact, non-injured retinas did not show any toxic effects (Fig. 8A, B; hatched bars). Furthermore, treatment with rolipram, a selective inhibitor of PDE4, resulted in a neuroprotective effect of both RGC soma and axons similar to that obtained with ibudilast (Fig. 8A, B; dark grey bars). Collectively, our data demonstrate that ibudilast mediates RGC survival through activation of the PKA pathway.

III.5. DISCUSSION

Despite accumulating evidence linking glial activation and neuroinflammation with glaucomatous damage, little is known about whether interfering with the inflammatory response is beneficial for neuronal viablity. In this regard, the data presented here, using a well characterized rat model of glaucoma reveal several novel findings. First, we show that ocular hypertension results in astrocyte reactivity, microgliosis and accumulation of pro-inflammatory cytokines, a response that is markedly attenuated by ibudilast. Second, we demonstrate that intraocular administration of ibudilast effectively protects RGC soma and axons in glaucomatous eyes. Third, we show that elevated IOP triggers PDE4A expression in Müller glia and that ibudilast leads to cAMP accumulation in target cells. Lastly, our results indicate that the effect of ibudilast on the glial cell response and subsequent RGC survival in glaucoma are dependent on PKA activation. This study identifies ibudilast as a useful glial cell modulator with favourable effects on neuronal viability in glaucoma, and reveals that the cAMP/PKA pathway is a key regulator of this response. Collectively, our data suggest that attenuation of some aspects of the inflammatory response during glaucoma, including overt glial reactivity, might be helpful to enhance viability of neurons subjected to chronic ocular hypertension.

A prominent neuroinflammatory component, reported in glaucomatous human retinas and in animal models of the disease, has been proposed to contribute to RGC damage (Soto and Howell 2014b). This inflammatory response is mediated by reactive astrocytes, Müller cells, microglia, and infiltrating monocytes acting in a coordinated manner (Chong and Martin 2015). Astrocytes form a cellular network in close association with the retinal vasculature, where they regulate the blood-retinal interface. In the rodent optic nerve head, GFAP-positive astrocytes form a dense meshwork that ensheaths RGC axons organizing them into fiber bundles (Sun et al.

2009). Recent data demonstrate that astrocytes in the optic nerve head are endowed with a constitutive phagocytic activity and engulf mitochondria-containing RGC axon segments for degradation (Davis and Marsh-Armstrong 2014). Müller cells are specialized radial glia that span the entire thickness of the retina and have processes that closely wrap around neuronal cell bodies and dendrites. They provide metabolic support and regulate the retinal microenvironment (Bringmann et al. 2006), hence are ideally suited to control neuronal viability. Our data demonstrate that reactive astrocytes and Müller glia upregulate GFAP in the rat retina and optic nerve following induction of ocular hypertension. This is consistent with previous studies showing that both astrocytes and Müller cells rapidly upregulate GFAP and undergo important morphological changes characterized by cell body hypertrophy and loss of processes (Bringmann and Wiedemann 2009; Lye-Barthel et al. 2013; Varela and Hernandez 1997). Importantly, we show that intraocular administration of ibudilast markedly attenuated the number of GFAP-positive astrocytes and Müller cell processes, as well as the overall levels of GFAP expression, demonstrating the ability of ibudilast as a glial cell modulator *in vivo*.

Retinal and optic nerve microglia represent a self-renewing population of innate immune cells that perform surveillance activities, monitor their environment and clear away metabolic products and tissue debris (Cuenca et al. 2014). Recent studies using DBA/2J mice, a strain that develops spontaneous age-related glaucoma, indicate that microgliosis occurs at early stages of the disease and that the level of microglia activation correlates with the severity of neurodegeneration (Bosco et al. 2015; Bosco et al. 2011). Our data demonstrate a substantial increase in the density of Iba1-positive cells in the retina and optic nerve of rats subjected to ocular hypertension, which was accompanied by morphological changes characterized by reduced ramifications and rounded morphology suggestive of an activated state. We also

observed increased CD68 expression in Iba1-positive cells, which has been correlated with increased phagocytic activity (Penfold et al. 1991). These findings are consistent with previous studies showing proliferation of microglia after optic nerve injury (Wohl et al. 2010) and the presence of CD68-positive cells with amoeboid morphology in rodent models of ocular hypertension (Ebneter et al. 2010; Rojas et al. 2014). Ibudilast effectively decreased the density of Iba1/CD68-positive cells suggesting that it reduces proliferation and activation of microglia/macrophages in glaucoma.

Reactive glia are the primary source of pro-inflammatory cytokines in the CNS. Our data show that TNFα, IL-1β, IL-6 and MIF are markedly upregulated during ocular hypertension. Importantly, ibudilast-mediated attenuation of glial cell reactivity correlated with a significant decrease in the levels of these inflammatory factors. Among these, TNFα plays a prominent role in glaucomatous neurodegeneration. For example, exogenous TNFα promotes death of RGCs (Lebrun-Julien et al. 2009a; Nakazawa et al. 2006) and lack of TNFR1 protects these neurons from mechanical injury (Tezel et al. 2004). High-throughput characterization of the retinal proteome revealed prominent upregulation of TNFα/TNFR1 signaling in human glaucoma (Yang et al. 2011b). Furthermore, TNFα levels are elevated in aqueous humor samples from glaucoma patients (Balaiya et al. 2011; Sawada et al. 2010), and TNFα gene polymorphisms are associated with primary open angle glaucoma (Xin et al. 2013). Of interest, a recent study demonstrated that soluble TNFα promotes RGC death by increasing cell surface expression of calcium permeable AMPA receptors (Cueva Vargas JL. 2015). Although the role of IL-1β, IL-6 and MIF is not well understood in the context of glaucoma, there is evidence suggesting that the levels of expression of these cytokines are modulated by retinal and optic nerve damage. IL-1β and IL-6 have been shown to be upregulated by retinal microglia during ischemia-reperfusion injury (Sanchez et al.

2003) and after optic nerve crush injury or induction of ocular hypertension (Chidlow et al. 2012; Leibinger et al. 2013). In the retina, MIF is expressed by astrocytes and Müller cells (Matsuda et al. 1997). MIF has been shown to contribute to the pathogenesis of Alzheimer's disease and experimental autoimmune encephalomyelitis (Bacher et al. 2010; Popp et al. 2009), and to promote neuronal death in experimental stroke (Inacio et al. 2011). Based on this, it is plausible that the ability of ibudilast to confer robust RGC survival in glaucoma is partly mediated by a reduction of glia-derived TNFα and possibly other pro-inflammatory cytokines.

Recent evidence indicates that PDEs are important regulators of inflammation in the CNS (Paterniti et al. 2014). PDEs are classified depending on their substrate specificity, location and structure (Charbonneau et al. 1986; Mehats et al. 2002). Among these, PDE4 is considered of particular functional relevance due to its abundance in neural tissue (Iona et al. 1998). Ibudilast is a cAMP PDE inhibitor with high affinity for PDE4 (Huang et al. 2006) , and PDE4 is abundantly expressed by retinal cells (Whitaker and Cooper 2009). Our data demonstrate that PDE4A, B and D are expressed in many cell-types in the inner nuclear and ganglion cell layers, including RGCs. Of interest, we found that ocular hypertension triggered a noticeable increase of PDE4A in Müller glia, which correlated with detectable accumulation of cAMP in these cells in the presence of ibudilast. This finding suggests that Müller glia are likely to be an important cellular target of ibudilast action. Given that RGCs express constitutive levels of PDE4A, it is also possible that these neurons are directly affected by ibudilast which can result in further increase of endogenous cAMP. Intriguingly, in spite of the robust response elicited in microglia/macrophages by ibudilast, we did not detect PDE4A expression in Iba1-positive cells suggesting that PDE4A in microglia is below detection levels or that microglia respond indirectly through dynamic cross-talk with Müller cells. Indeed, Müller cells have been shown to

signal microglia via the translocator protein (TSPO) to regulate microglial activity and hence modulate the resolution of inflammation (Wang et al. 2014).

How does ibudilast attenuate glial reactivity and promote neuronal survival in glaucoma? Our data suggest a scenario in which ibudilast inhibits PDE4 in target cells leading to increased cAMP levels and PKA activation. This hypothesis is supported by our finding that co-administration of ibudilast with the PKA inhibitor Rp-cAMPS completely obliterated the neuroprotective effect of ibudilast alone. Furthermore, the observation that rolipram, a selective inhibitor of PDE4, promoted a survival effect similar to that of ibudilast supports a crucial role for PDE4. Nonetheless, it is conceivable that the activation of the cAMP/PKA pathway might have distinct responses in glia versus neurons. While increased cAMP and PKA activity is likely to reduce glial cell reactivity and production of pro-inflammatory cytokines, the same response might stimulate survival pathways in injured neurons. For example, low cAMP levels and reduced PKA activity has been attributed to an interaction between PDE4A and $p75^{NTR}$ (Sachs and Akassoglou 2007), a death receptor solely expressed by Müller cells in the adult retina (Lebrun-Julien et al. 2010; Lebrun-Julien et al. 2009b). Indeed, $p75^{NTR}$ activation stimulates nuclear factor κB (NF-κB) and TNFα production by Müller cells (Lebrun-Julien et al. 2010), and $p75^{NTR}$ inhibition protects RGCs from axonal injury-induced death (Lebrun-Julien et al. 2009b). When ibudilast inhibits PDE4A, the inflammatory process is reversed by increasing cAMP levels, which has been shown to inhibit NF-κB thus switching off gene expression of numerous inflammatory and immune mediators (Gerlo et al. 2011). In contrast, increased cAMP/PKA signaling in RGCs has been associated with neuronal survival, axonal growth, electrical activity, enhanced responsiveness to neurotrophic factors and synaptogenesis (Corredor et al. 2012; Cui et al. 2003; Firth et al. 2005; Goldberg et al. 2002; Li et al. 2003; Meyer-Franke et al. 1998;

Stellwagen et al. 1999; Watanabe et al. 2003). By increasing the levels of cAMP in RGCs, ibudilast activates PKA which in turn might stimulate the cAMP regulatory element-binding protein (CREB) (Fujino et al. 2009; Mali et al. 2011; Park et al. 2015) thus triggering a transcriptional program that enhances neuronal survival in glaucoma.

In conclusion, our study identifies ibudilast, a safe and well-tolerated glial cell modulator, to effectively attenuate gliosis and production of pro-inflammatory mediators while enhancing neuronal viability in glaucoma. Our data support a model in which ibudilast promotes RGC survival through inhibition of PDE4 activity leading to increased cAMP levels and subsequent PKA activation. This study sheds light on the potential utility of modulators of the PDE4/cAMP/PKA pathway to counter the damaging effects of the inflammatory response in glaucomatous neurodegeneration.

III.6. REFERENCES

Ahmed F, Brown KM, Stephan DA, Morrison JC, Johnson EC, Tomarev SI. 2004. Microarray analysis of changes in mRNA levels in the rat retina after experimental elevation of intraocular pressure. Invest Ophthalmol Vis Sci 45(4):1247-58.

Bacher M, Deuster O, Aljabari B, Egensperger R, Neff F, Jessen F, Popp J, Noelker C, Reese JP, Al-Abed Y and others. 2010. The role of macrophage migration inhibitory factor in Alzheimer's disease. Mol Med 16(3-4):116-21.

Balaiya S, Edwards J, Tillis T, Khetpa lV, Chalam KV. 2011. Tumor necrosis factor-alpha (TNF-α) levels in aqueous humor of primary open angle glaucoma. Clin Ophthalmol 5:553-556.

Bosco A, Romero CO, Breen KT, Chagovetz AA, Steele MR, Ambati BK, Vetter ML. 2015. Neurodegeneration severity is anticipated by early microglia alterations monitored in vivo in a mouse model of chronic glaucoma. Dis Model Mech.

Bosco A, Steele MR, Vetter ML. 2011. Early microglia activation in a mouse model of chronic glaucoma. J Comp Neurol 519(4):599-620.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A. 2006. Muller cells in the healthy and diseased retina. Prog Retin Eye Res 25(4):397-424.

Bringmann A, Wiedemann P. 2009. Involvement of Muller glial cells in epiretinal membrane formation. Graefes Arch Clin Exp Ophthalmol 247(7):865-83.

Charbonneau H, Beier N, Walsh KA, Beavo JA. 1986. Identification of a conserved domain among cyclic nucleotide phosphodiesterases from diverse species. Proc Natl Acad Sci U S A 83(24):9308-12.

Chidlow G, Wood JP, Ebneter A, Casson RJ. 2012. Interleukin-6 is an efficacious marker of axonal transport disruption during experimental glaucoma and stimulates neuritogenesis in cultured retinal ganglion cells. Neurobiol Dis 48(3):568-81.

Chong RS, Martin KR. 2015. Glial cell interactions and glaucoma. Curr Opin Ophthalmol 26(2):73-7.

Corredor RG, Trakhtenberg EF, Pita-Thomas W, Jin X, Hu Y, Goldberg JL. 2012. Soluble adenylyl cyclase activity is necessary for retinal ganglion cell survival and axon growth. J Neurosci 32(22):7734-44.

Cuenca N, Fernandez-Sanchez L, Campello L, Maneu V, De la Villa P, Lax P, Pinilla I. 2014. Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. Prog Retin Eye Res 43:17-75.

Cueva Vargas JL, Osswald IK, Unsain N, Aurousseau MR, Barker PA, Bowie D, Di Polo A. 2015. Soluble Tumor Necrosis Factor Alpha Promotes Retinal Ganglion Cell Death in Glaucoma via Calcium-Permeable AMPA Receptor Activation. J Neurosci 35(35):12088-102.

Cueva Vargas JL. OI, Unsain N, Aurousseau M, Barker P, Bowie D, and Di Polo A. 2015. Soluble tumor necrosis factor alpha promotes retinal ganglion cell death in glaucoma via calcium-permeable AMPA receptor activation. J Neuroscience Under revision.

Cui Q, Yip HK, Zhao RC, So KF, Harvey AR. 2003. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. Mol Cell Neurosci 22(1):49-61.

Davis CH, Marsh-Armstrong N. 2014. Discovery and implications of transcellular mitophagy. Autophagy 10(12):2383-4.
Dostmann WR. 1995. (RP)-cAMPS inhibits the cAMP-dependent protein kinase by blocking the cAMP-induced conformational transition. FEBS Lett 375(3):231-4.
Ebneter A, Casson RJ, Wood JP, Chidlow G. 2010. Microglial activation in the visual pathway in experimental glaucoma: spatiotemporal characterization and correlation with axonal injury. Invest Ophthalmol Vis Sci 51(12):6448-60.
Firth SI, Wang CT, Feller MB. 2005. Retinal waves: mechanisms and function in visual system development. Cell Calcium 37(5):425-32.
Fujino H, Kitaoka Y, Hayashi Y, Munemasa Y, Takeda H, Kumai T, Kobayashi S, Ueno S. 2009. Axonal protection by brain-derived neurotrophic factor associated with CREB phosphorylation in tumor necrosis factor-alpha-induced optic nerve degeneration. Acta Neuropathol 117(1):75-84.
Gerlo S, Kooijman R, Beck IM, Kolmus K, Spooren A, Haegeman G. 2011. Cyclic AMP: a selective modulator of NF-kappaB action. Cell Mol Life Sci 68(23):3823-41.
Gibson LC, Hastings SF, McPhee I, Clayton RA, Darroch CE, Mackenzie A, Mackenzie FL, Nagasawa M, Stevens PA, Mackenzie SJ. 2006. The inhibitory profile of Ibudilast against the human phosphodiesterase enzyme family. Eur J Pharmacol 538(1-3):39-42.
Goldberg JL, Espinosa JS, Xu Y, Davidson N, Kovacs GT, Barres BA. 2002. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron 33(5):689-702.
Hernandez MR, Miao H, Lukas T. 2008. Astrocytes in glaucomatous optic neuropathy. Prog Brain Res 173:353-73.
Houslay MD, Schafer P, Zhang KY. 2005. Keynote review: phosphodiesterase-4 as a therapeutic target. Drug Discov Today 10(22):1503-19.
Howell GR, Macalinao DG, Sousa GL, Walden M, Soto I, Kneeland SC, Barbay JM, King BL, Marchant JK, Hibbs M and others. 2011. Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. J Clin Invest 121(4):1429-44.
Howell GR, Soto I, Zhu X, Ryan M, Macalinao DG, Sousa GL, Caddle LB, MacNicoll KH, Barbay JM, Porciatti V and others. 2012. Radiation treatment inhibits monocyte entry into the optic nerve head and prevents neuronal damage in a mouse model of glaucoma. J Clin Invest 122(4):1246-61.
Huang Z, Liu S, Zhang L, Salem M, Greig GM, Chan CC, Natsumeda Y, Noguchi K. 2006. Preferential inhibition of human phosphodiesterase 4 by ibudilast. Life Sci 78(23):2663-8.
Imai Y, Kohsaka S. 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. Glia 40(2):164-74.
Inacio AR, Ruscher K, Leng L, Bucala R, Deierborg T. 2011. Macrophage migration inhibitory factor promotes cell death and aggravates neurologic deficits after experimental stroke. J Cereb Blood Flow Metab 31(4):1093-106.
Inman DM, Horner PJ. 2007. Reactive nonproliferative gliosis predominates in a chronic mouse model of glaucoma. Glia 55(9):942-53.
Iona S, Cuomo M, Bushnik T, Naro F, Sette C, Hess M, Shelton ER, Conti M. 1998. Characterization of the rolipram-sensitive, cyclic AMP-specific phosphodiesterases:

identification and differential expression of immunologically distinct forms in the rat brain. Mol Pharmacol 53(1):23-32.
Johnson EC, Doser TA, Cepurna WO, Dyck JA, Jia L, Guo Y, Lambert WS, Morrison JC. 2011. Cell proliferation and interleukin-6-type cytokine signaling are implicated by gene expression responses in early optic nerve head injury in rat glaucoma. Invest Ophthalmol Vis Sci 52(1):504-18.
Johnson EC, Morrison JC. 2009. Friend or foe? Resolving the impact of glial responses in glaucoma. J Glaucoma 18(5):341-53.
Kuehn MH, Kim CY, Jiang B, Dumitrescu AV, Kwon YH. 2008. Disruption of the complement cascade delays retinal ganglion cell death following retinal ischemia-reperfusion. Exp Eye Res 87(2):89-95.
Lebrun-Julien F, Bertrand MJ, De Backer O, Stellwagen D, Morales CR, Di Polo A, Barker PA. 2010. ProNGF induces TNFalpha-dependent death of retinal ganglion cells through a p75NTR non-cell-autonomous signaling pathway. Proc Natl Acad Sci U S A 107(8):3817-22.
Lebrun-Julien F, Duplan L, Pernet V, Osswald IK, Sapieha P, Bourgeois P, Dickson K, Bowie D, Barker PA, Di Polo A. 2009a. Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. J Neurosci 29(17):5536-5545.
Lebrun-Julien F, Morquette B, Douillette A, Saragovi HU, Di Polo A. 2009b. Inhibition of p75(NTR) in glia potentiates TrkA-mediated survival of injured retinal ganglion cells. Mol Cell Neurosci 40(4):410-20.
Leibinger M, Muller A, Gobrecht P, Diekmann H, Andreadaki A, Fischer D. 2013. Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell Death Dis 4:e609.
Li Y, Irwin N, Yin Y, Lanser M, Benowitz LI. 2003. Axon regeneration in goldfish and rat retinal ganglion cells: differential responsiveness to carbohydrates and cAMP. J Neurosci 23(21):7830-8.
Lye-Barthel M, Sun D, Jakobs TC. 2013. Morphology of astrocytes in a glaucomatous optic nerve. Invest Ophthalmol Vis Sci 54(2):909-17.
Mali RS, Zhang XM, Chintala SK. 2011. A decrease in phosphorylation of cAMP-response element-binding protein (CREBP) promotes retinal degeneration. Exp Eye Res 92(6):528-36.
Matsuda A, Tagawa Y, Yoshida K, Matsuda H, Nishihira J. 1997. Expression of macrophage migration inhibitory factor in rat retina and its immunohistochemical localization. J Neuroimmunol 77(1):85-90.
Mehats C, Andersen CB, Filopanti M, Jin SL, Conti M. 2002. Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. Trends Endocrinol Metab 13(1):29-35.
Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, Hanson MG, Jr., Reichardt LF, Barres BA. 1998. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21(4):681-93.
Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, Suzumura A. 2004. Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 46(3):404-11.
Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. 1997. A rat model of chronic pressure-induced optic nerve damage. Exp Eye Res 64(1):85-96.

Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, Salinas-Navarro M, Vidal-Sanz M, Agudo M. 2009. Brn3a as a marker of retinal ganglion cells: qualitative and quantitative time course studies in naive and optic nerve-injured retinas. Invest Ophthalmol Vis Sci 50(8):3860-8.

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI. 2006. Tumor necrosis factor-{alpha} mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J Neurosci 26(49):12633-12641.

Park YH, Mueller BH, 2nd, McGrady NR, Ma HY, Yorio T. 2015. AMPA receptor desensitization is the determinant of AMPA receptor mediated excitotoxicity in purified retinal ganglion cells. Exp Eye Res 132:136-50.

Paterniti I, Esposito E, Cuzzocrea S. 2014. Phosphodiesterase as a new therapeutic target for the treatment of spinal cord injury and neurodegenerative diseases. Curr Med Chem 21(24):2830-8.

Penfold PL, Madigan MC, Provis JM. 1991. Antibodies to human leucocyte antigens indicate subpopulations of microglia in human retina. Vis Neurosci 7(4):383-8.

Popp J, Bacher M, Kolsch H, Noelker C, Deuster O, Dodel R, Jessen F. 2009. Macrophage migration inhibitory factor in mild cognitive impairment and Alzheimer's disease. J Psychiatr Res 43(8):749-53.

Ren L, Danias J. 2010. A role for complement in glaucoma? Adv Exp Med Biol 703:95-104.

Rodgers KM, Deming YK, Bercum FM, Chumachenko SY, Wieseler JL, Johnson KW, Watkins LR, Barth DS. 2014. Reversal of established traumatic brain injury-induced, anxiety-like behavior in rats after delayed, post-injury neuroimmune suppression. J Neurotrauma 31(5):487-97.

Rojas B, Gallego BI, Ramirez AI, Salazar JJ, de Hoz R, Valiente-Soriano FJ, Aviles-Trigueros M, Villegas-Perez MP, Vidal-Sanz M, Trivino A and others. 2014. Microglia in mouse retina contralateral to experimental glaucoma exhibit multiple signs of activation in all retinal layers. J Neuroinflammation 11:133.

Rolan P, Hutchinson M, Johnson K. 2009. Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. Expert Opin Pharmacother 10(17):2897-904.

Sachs BD, Akassoglou K. 2007. Regulation of cAMP by the p75 neurotrophin receptor: insight into drug design of selective phosphodiesterase inhibitors. Biochem Soc Trans 35(Pt 5):1273-7.

Sanchez-Guajardo V, Febbraro F, Kirik D, Romero-Ramos M. 2010. Microglia acquire distinct activation profiles depending on the degree of alpha-synuclein neuropathology in a rAAV based model of Parkinson's disease. PLoS One 5(1):e8784.

Sanchez RN, Chan CK, Garg S, Kwong JM, Wong MJ, Sadun AA, Lam TT. 2003. Interleukin-6 in retinal ischemia reperfusion injury in rats. Invest Ophthalmol Vis Sci 44(9):4006-11.

Sawada H, Fukuchi T, Tanaka T, Abe H. 2010. Tumor necrosis factor- α concentrations in the aqueous humor of patients with glaucoma. Invest Ophthalmol Vis Sci 51(2):903-906.

Schwartz M, Baruch K. 2014. Breaking peripheral immune tolerance to CNS antigens in neurodegenerative diseases: boosting autoimmunity to fight-off chronic neuroinflammation. J Autoimmun 54:8-14.

Schwartz M, London A. 2008. Glaucoma as a neuropathy amenable to neuroprotection and immune manipulation. Prog Brain Res 173:375-84.

Schwartz M, London A. 2009. Immune maintenance in glaucoma: boosting the body's own neuroprotective potential. J Ocul Biol Dis Infor 2(2):73-77.
Scott JD. 1991. Cyclic nucleotide-dependent protein kinases. Pharmacol Ther 50(1):123-45.
Soto I, Howell GR. 2014a. The complex role of neuroinflammation in glaucoma. Cold Spring Harbor Perspectives in Medicine.
Soto I, Howell GR. 2014b. The complex role of neuroinflammation in glaucoma. Cold Spring Harb Perspect Med 4(8).
Stasi K, Nagel D, Yang X, Wang RF, Ren L, Podos SM, Mittag T, Danias J. 2006. Complement component 1Q (C1Q) upregulation in retina of murine, primate, and human glaucomatous eyes. Invest Ophthalmol Vis Sci 47(3):1024-9.
Stellwagen D, Shatz CJ, Feller MB. 1999. Dynamics of retinal waves are controlled by cyclic AMP. Neuron 24(3):673-85.
Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B and others. 2007. The classical complement cascade mediates CNS synapse elimination. Cell 131(6):1164-78.
Sun D, Lye-Barthel M, Masland RH, Jakobs TC. 2009. The morphology and spatial arrangement of astrocytes in the optic nerve head of the mouse. J Comp Neurol 516(1):1-19.
Sun D, Qu J, Jakobs TC. 2013. Reversible reactivity by optic nerve astrocytes. Glia 61(8):1218-35.
Suzumura A, Ito A, Yoshikawa M, Sawada M. 1999. Ibudilast suppresses TNFalpha production by glial cells functioning mainly as type III phosphodiesterase inhibitor in the CNS. Brain Res 837(1-2):203-12.
Tezel G. 2013. Immune regulation toward immunomodulation for neuroprotection in glaucoma. Current Opinion in Pharmacology 13(1):23-31.
Tezel G, Yang X, Luo C, Cai J, Powell DW. 2012. An astrocyte-specific proteomic approach to inflammatory responses in experimental rat glaucoma. Invest Ophthalmol Vis Sci 53(7):4220-33.
Tezel G, Yang X, Luo C, Kain AD, Powell DW, Kuehn MH, Kaplan HJ. 2010. Oxidative stress and the regulation of complement activation in human glaucoma. Invest Ophthalmol Vis Sci 51(10):5071-82.
Tezel G, Yang X, Yang J, Wax MB. 2004. Role of tumor necrosis factor receptor-1 in the death of retinal ganglion cells following optic nerve crush injury in mice. Brain Res 996(2):202-212.
Tham YC, Li X, Wong TY, Quigley HA, Aung T, Cheng CY. 2014. Global Prevalence of Glaucoma and Projections of Glaucoma Burden through 2040: A Systematic Review and Meta-Analysis. Ophthalmology 121(11):2081-90.
Travaglione S, Falzano L, Fabbri A, Stringaro A, Fais S, Fiorentini C. 2002. Epithelial cells and expression of the phagocytic marker CD68: scavenging of apoptotic bodies following Rho activation. Toxicol In Vitro 16(4):405-11.
Varela HJ, Hernandez MR. 1997. Astrocyte responses in human optic nerve head with primary open-angle glaucoma. J Glaucoma 6(5):303-13.
Wang L, Cioffi GA, Cull G, Dong J, Fortune B. 2002. Immunohistologic evidence for retinal glial cell changes in human glaucoma. Invest Ophthalmol Vis Sci 43(4):1088-94.
Wang M, Wang X, Zhao L, Ma W, Rodriguez IR, Fariss RN, Wong WT. 2014. Macroglia-microglia interactions via TSPO signaling regulates microglial activation in the mouse retina. J Neurosci 34(10):3793-806.

Watanabe M, Tokita Y, Kato M, Fukuda Y. 2003. Intravitreal injections of neurotrophic factors and forskolin enhance survival and axonal regeneration of axotomized beta ganglion cells in cat retina. Neuroscience 116(3):733-42.

Weinreb RN, Khaw PT. 2004. Primary open-angle glaucoma. Lancet 363(9422):1711-20.

Whitaker CM, Cooper NG. 2009. The novel distribution of phosphodiesterase-4 subtypes within the rat retina. Neuroscience 163(4):1277-91.

Wohl SG, Schmeer CW, Witte OW, Isenmann S. 2010. Proliferative response of microglia and macrophages in the adult mouse eye after optic nerve lesion. Invest Ophthalmol Vis Sci 51(5):2686-96.

Xin X, Gao L, Wu T, Sun F. 2013. Roles of tumor necrosis factor alpha gene polymorphisms, tumor necrosis factor alpha level in aqueous humor, and the risks of open angle glaucoma: a meta-analysis. Mol Vis 19:526-535.

Yang X, Luo C, Cai J, Powell DW, Yu D, Kuehn MH, Tezel G. 2011a. Neurodegenerative and inflammatory pathway components linked to TNF-alpha/TNFR1 signaling in the glaucomatous human retina. Invest Ophthalmol Vis Sci 52(11):8442-54.

Yang X, Luo C, Cai J, Powell DW, Yu D, Kuehn MH, Tezel G. 2011b. Neurodegenerative and Inflammatory Pathway Components Linked to TNF-α/TNFR1 Signaling in the Glaucomatous Human Retina. Invest Ophthalmol & Vis Sci 52(11):8442-8454.

Yuan L, Neufeld AH. 2001. Activated microglia in the human glaucomatous optic nerve head. J Neurosci Res 64(5):523-32.

Figure 1. Ibudilast attenuates astrocyte reactivity in experimental glaucoma.

A, D, and G) Basal level of GFAP labeling was detected in astrocytic processes in the retinal GCL, as well as astrocytes in the optic nerve head and optic nerve proper of the non-injured eyes. **B)** Three weeks after induction of ocular hypertension, high levels of GFAP was observed in retinal astrocytes as well as the proximal Müller cell radial processes. **E and H)** GFAP labeling was upregulated in astrocytes residing in the optic nerve. **C, F and I)** Intraocular injection of ibudilast attenuated GFAP immunostaining both in the retina and optic nerve of glaucomatous eyes. **J-K)** Western blot analysis showed an increase in GFAP protein levels in retinas exposed to ocular hypertension compared to intact controls. Ibudilast treatment resulted in a substantial decrease of retinal GFAP expression in glaucoma. Values are expressed as mean ± S.E.M. (ANOVA, *=$p<0.05$, **$p=<0.01$; Tukey's multiple comparison test). ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars: A-C= 20 μm, D-I= 100 μm.

Figure 2. Retinal microgliosis is reduced by ibudilast.

A-B) Retinal cross section showed an increase in the number of microglia/macrophages in the glaucomatous eye using Iba1. **D-E)** Flat-mounted retinas showed reduced ramifications in Iba1-positive cells during ocular hypertension. **C, F)** Ibudilast treatment reduced the number of Iba1-positive cells. **G-O)** Staining of flat-mounted retinas with Iba1 and CD68, demonstrated a substantial increase in the number of double-labeled cells, which was markedly reduced in the presence of ibudilast. **P)** Quantitative analysis confirmed the increase in the number of CD68/Iba1-positive cells compared to intact retinas, and that ibudilast treatment significantly attenuated this response. Values are expressed as mean ± S.E.M. (ANOVA, *$p<0.05$; Tukey's multiple comparison test). Values are expressed as mean ± S.E.M. (ANOVA, *$p<0.05$; Tukey's multiple comparison test). ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 50 μm.

Figure 3. Activation of microglia/macrophages in the optic nerve is reduced by ibudilast.

A-F) Optic nerve at the levels of its head and proximal segment demonstrated an increase in the number of Iba1-positive cells after induction of ocular hypertension, which was effectively reduced with ibudilast treatment. **G-O)** Double labeling with Iba1 and CD68 revealed an increase of active microglia/macrophages in glaucomatous eyes, which was reduced by ibudilast. **P)** Quantitative analysis confirmed an increase in CD68/Iba1-positive cells in optic nerves subjected to ocular hypertension, and a tangible decrease in the number of active microglia/macrophages with ibudilast. Values are expressed as mean ± S.E.M. (ANOVA, **$p<0.01$; Tukey's multiple comparison test). Scale bars: A-F = 100 μm, G-O = 20 μm.

Figure 4. Ibudilast attenuates the production of glial-derived pro-inflammatory cytokines in glaucoma.

Western blots analysis of **A)** TNFα, **C)** IL-1β, **E)** IL-6 and **G)** MIF from retinal homogenates showed that these cytokines are upregulated in glaucomatous retinas at three weeks after induction of ocular hypertension compared to the intact retina. This upregulation was significantly reduced by the intravitreal administration of ibudilast. We used β-actin to confirm equal protein loading. **B, D, F and H)** Densitometric analysis confirmed that ibudilast reduced the upregulation of these pro-inflammatory cytokines. Values are expressed as mean ± S.E.M. (ANOVA, *$p<0.05$, **$p<0.01$ and ***$p<0.001$; Tukey's multiple comparison test).

Figure 5. Ibudilast protects RGC soma and axon protection from ocular hypertension damage.

A-C) Flat-mounted retinas from eyes treated with ibudilast showed higher densities of cells positive for Brn3a, an RGC-specific marker(Nadal-Nicolas et al. 2009)(Nadal-Nicolas et al. 2009)(Nadal-Nicolas et al. 2009)(Nadal-Nicolas et al. 2009), compared to control retinas treated with vehicle (PBS). Scale bar: 50 μm. **D-F)** Analysis of optic nerve cross-sections showed a substantially larger number of RGC axon fibers with normal morphology in ibudilast-treated eyes compared to vehicle-treated controls. Scale bar: 10 μm. **G-H)** Quantitative analysis of RGC soma and axons confirmed that ibudilast-treated eyes (black bars) promoted significant RGCs soma and axons protection in experimental glaucoma compared to the vehicle-treated eyes (grey bars). Non-glaucomatous Brown Norway rat retinas are shown as reference (white bars, 100% survival). Values are expressed as mean ± S.E.M. (ANOVA, **p<0.01, ***p<0,001; Tukey's multiple comparison test).

Figure 6. Ocular hypertension triggers PDE4A expression in Müller cells.

A) Immunohistochemical analysis confirmed that PDE4A is expressed in many cells of the inner nuclear and ganglion cell layers in the intact, non-injured retina. **B-D)** PDE4A was expressed by RGCs, visualized with RBPMS. **E)** In glaucomatous retinas, PDE4A was presumptively detected in Müller glia. **F-H)** Co-localization of PDE4A with the Müller cell-specific marker Cellular Retinaldehyde Binding Protein (CRALBP) confirmed that ocular hypertension triggered PDE4A expression in these glial cells. **I-K)** PDE4A expression did not co-localize with Iba1-positive microglia/macrophages in glaucomatous retinas. **L-S)** PDE4B and PDE4D were also detected in cells of the inner nuclear and ganglion cell layers, including RGCs, but their expression pattern did not change with glaucoma (Fig. 6L-S). Scale bar= 20 μm.

Figure 7. Ibudilast treatment leads to cAMP accumulation in target cells.

A-C) Intraocular injection of ibudilast stimulated a marked increase in cAMP in glaucomatous retinas compared to vehicle-treated controls. **C-E)** Accumulation of cAMP was observed in Müller cell end-feet and radial processes, visualized with the specific marker GS. **F-H)** cAMP was also observed in RGCs, identified with RBPMS. Scale bars = 20 μm.

Figure 8. The neuroprotective effect of ibudilast is mediated by protein kinase A (PKA) activation.

A-B) Co-administration of ibudilast and Rp-cAMPS (light grey bars) completely blocked the pro-survival effect of ibudilast (black bars) on RGC soma and axons to levels found in vehicle-treated eyes. Injection of Rp-cAMPS into intact, non-injured retinas, did not show any toxic effects (hatched bars). Furthermore, treatment with rolipram, a selective inhibitor of PDE4, resulted in a neuroprotective effect of both RGC soma and axons similar to that obtained with ibudilast (dark grey bars). Intact retinas (white bars) represent 100% survival. Values are expressed as mean ± S.E.M. (ANOVA, **$p<0.01$ and *** $p<0.001$; Tukey's multiple comparison test).

III.7. TABLES

Table 1. Intraocular pressure (IOP) elevation in glaucomatous eyes treated with different compounds. Mean and peak IOP elevations for each cohort of rats exposed to 3 weeks of ocular hypertension (OHT) after treatment with ibudilast and Rp-cAMPS, or vehicle (n = 6-15/group).

Table 1. Intraocular pressure (IOP) elevation in glaucomatous eyes

Time after OHT surgery	Treatment	N	Mean IOP (mm Hg) ± S.E.M.			Peak IOP (mm Hg)	
			Glaucoma	Control	Difference	Glaucoma	Control
3 weeks	Vehicle	15	36.4 ± 0.62	24.4 ± 0.19	12.0 ± 0.53	40.0 ± 0.71	25.2 ± 1.16
	Ibudilast	15	36.6 ± 0.52	25.0 ± 0.23	11.6 ± 0.45	40.2 ± 1.77	26.6 ± 0.68
	Rp-cAMPS	6	36.0 ± 1.20	24.5 ± 0.42	11.4 ± 1.20	40.2 ± 2.18	25.6 ± 0.93

III.8. FIGURES

FIGURE 1

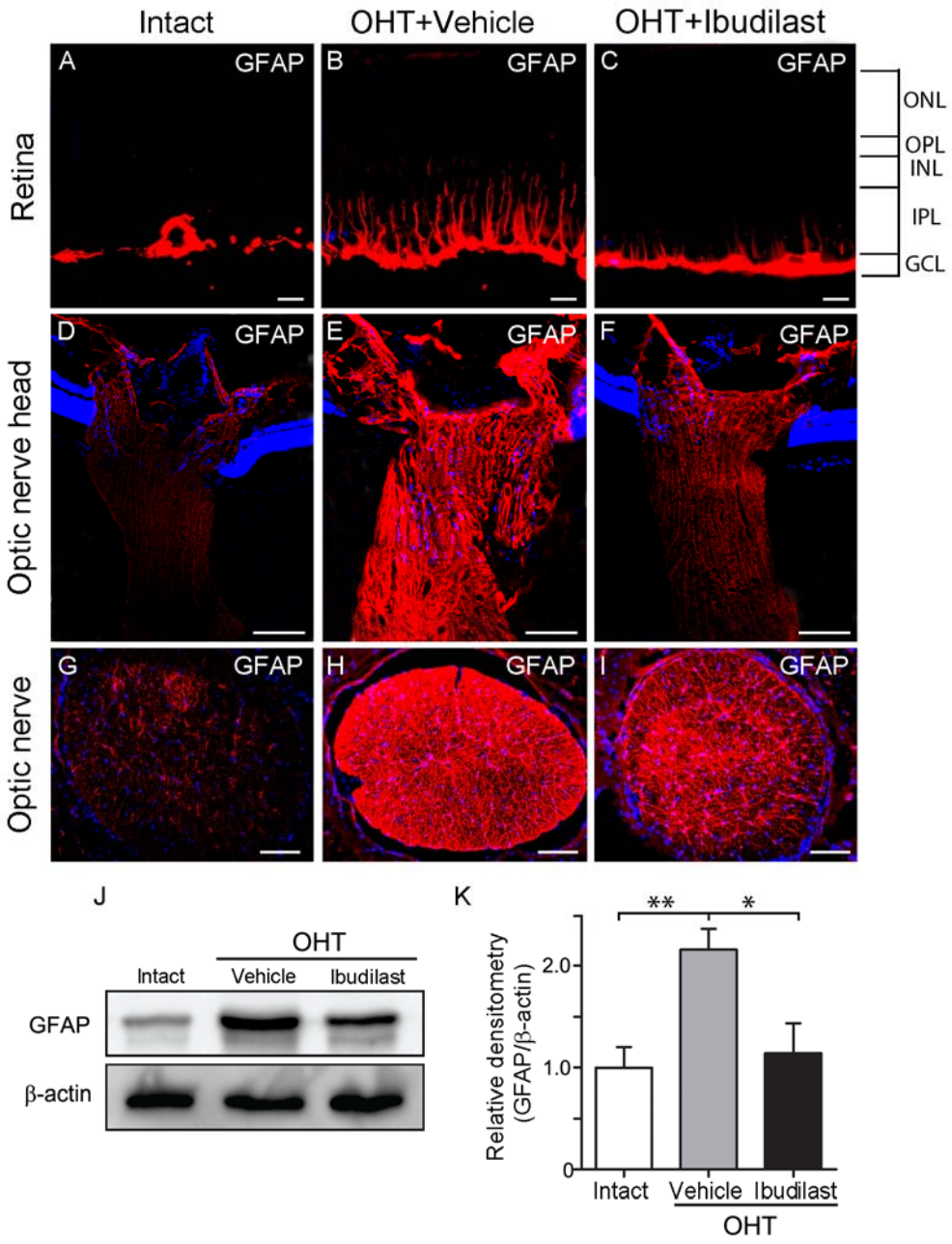


FIGURE 2

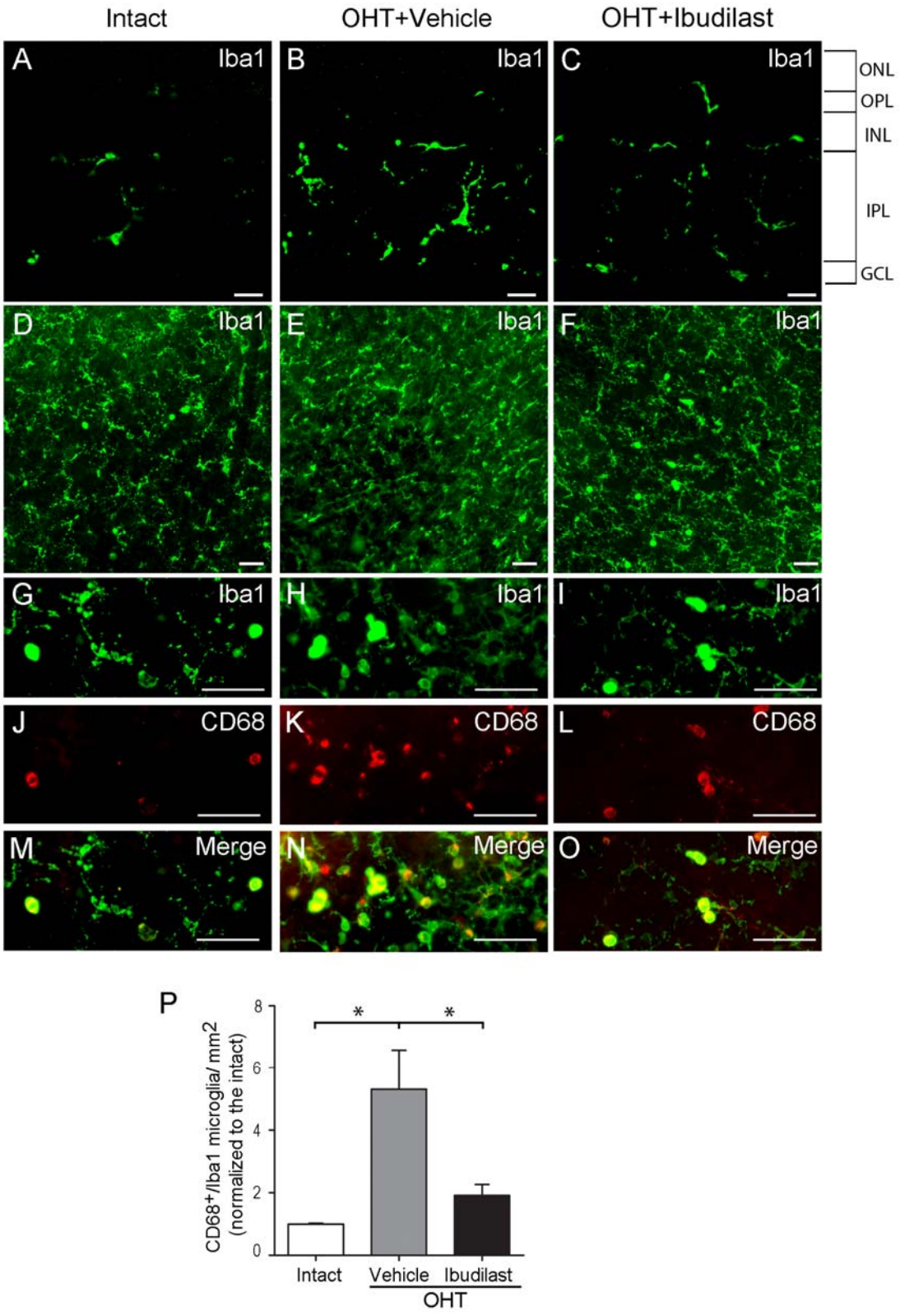

Intact
OHT+Vehicle
OHT+Ibudilast
A
Iba1
B
Iba1
C
Iba1
ONL
OPL
INL
IPL
GCL
D
Iba1
E
Iba1
F
Iba1
G
Iba1
H
Iba1
I
Iba1
J
CD68
K
CD68
L
CD68
M
Merge
N
Merge
O
Merge
P
CD68+/Iba1 microglia/ mm2
(normalized to the intact)
8
6
4
2
0
*
*
Intact
Vehicle
Ibudilast
OHT

FIGURE 3

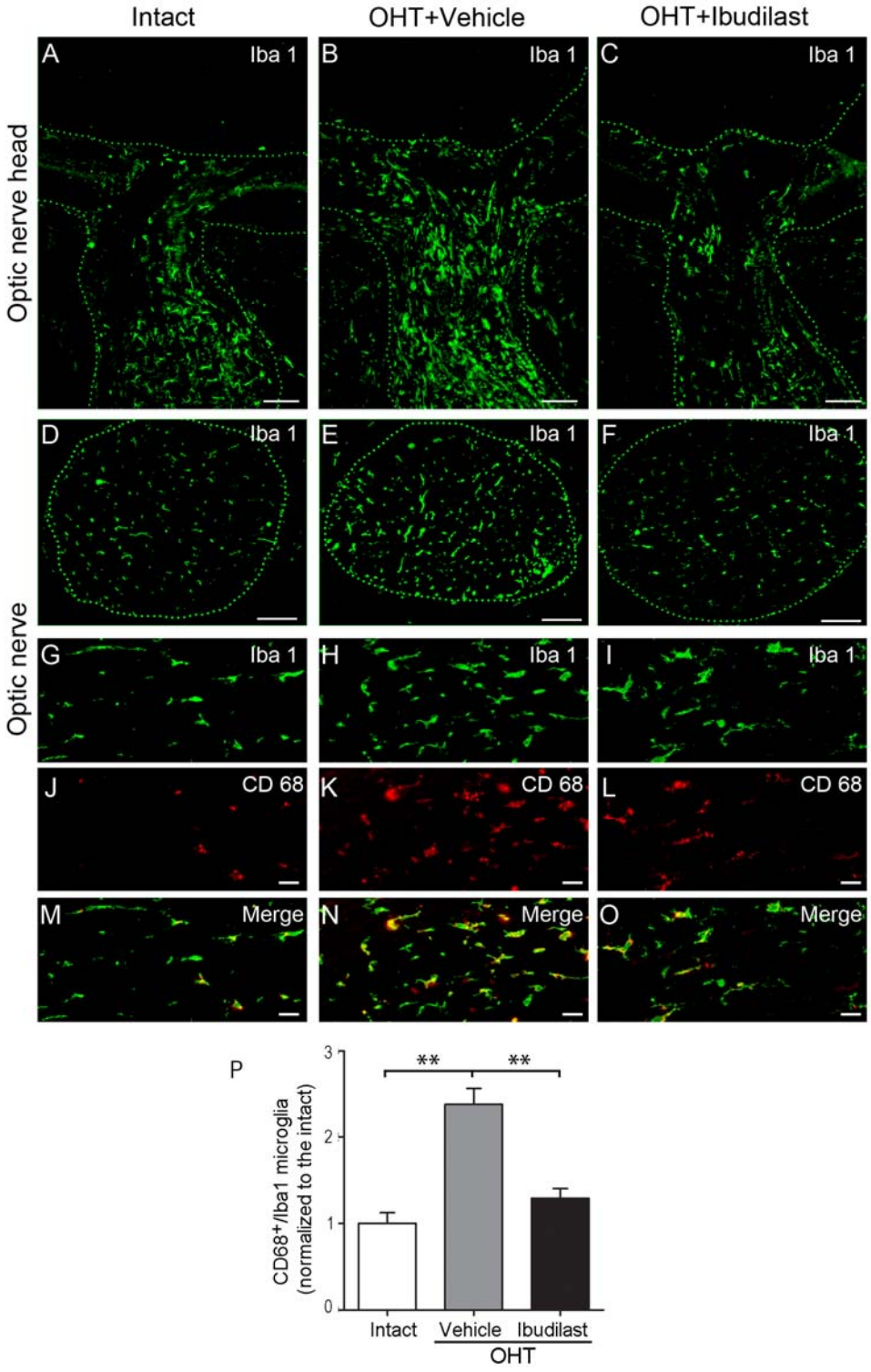

Intact
OHT+Vehicle
OHT+Ibudilast
Optic nerve head
Optic nerve
A
Iba 1
B
Iba 1
C
Iba 1
D
Iba 1
E
Iba 1
F
Iba 1
G
Iba 1
H
Iba 1
I
Iba 1
J
CD 68
K
CD 68
L
CD 68
M
Merge
N
Merge
O
Merge
P
CD68+/Iba1 microglia (normalized to the intact)
**
**
0
1
2
3
Intact
Vehicle
Ibudilast
OHT

FIGURE 4

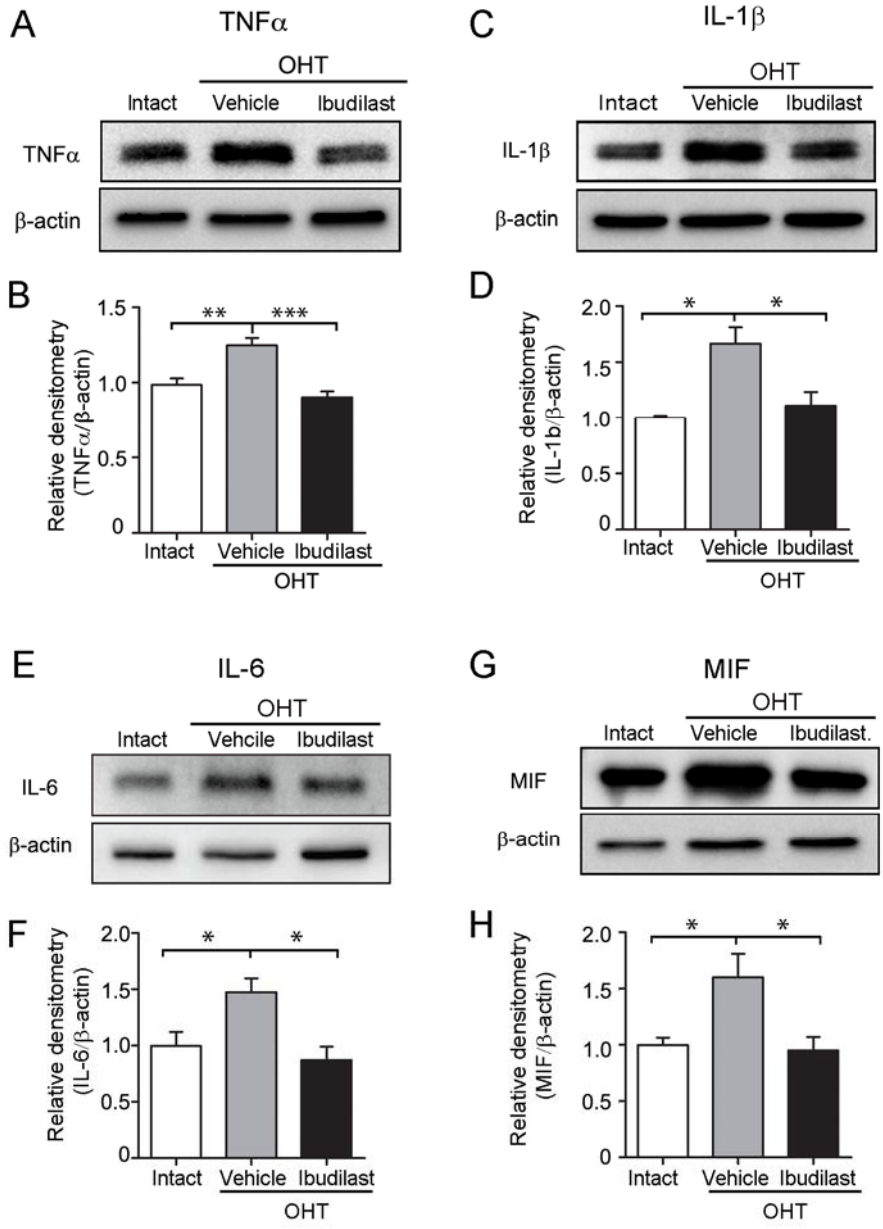

A
TNFα
OHT
Intact
Vehicle
Ibudilast
TNFα
β-actin
B
Relative densitometry (TNFα/β-actin)
1.5
1.0
0.5
0
**
***
Intact
Vehicle
Ibudilast
OHT
C
IL-1β
OHT
Intact
Vehicle
Ibudilast
IL-1β
β-actin
D
Relative densitometry (IL-1b/β-actin)
2.0
1.5
1.0
0.5
0
*
*
Intact
Vehicle
Ibudilast
OHT
E
IL-6
OHT
Intact
Vehcile
Ibudilast
IL-6
β-actin
F
Relative densitometry (IL-6/β-actin)
2.0
1.5
1.0
0.5
0
*
*
Intact
Vehicle
Ibudilast
OHT
G
MIF
OHT
Intact
Vehicle
Ibudilast.
MIF
β-actin
H
Relative densitometry (MIF/β-actin)
2.0
1.5
1.0
0.5
0
*
*
Intact
Vehicle
Ibudilast
OHT

FIGURE 5

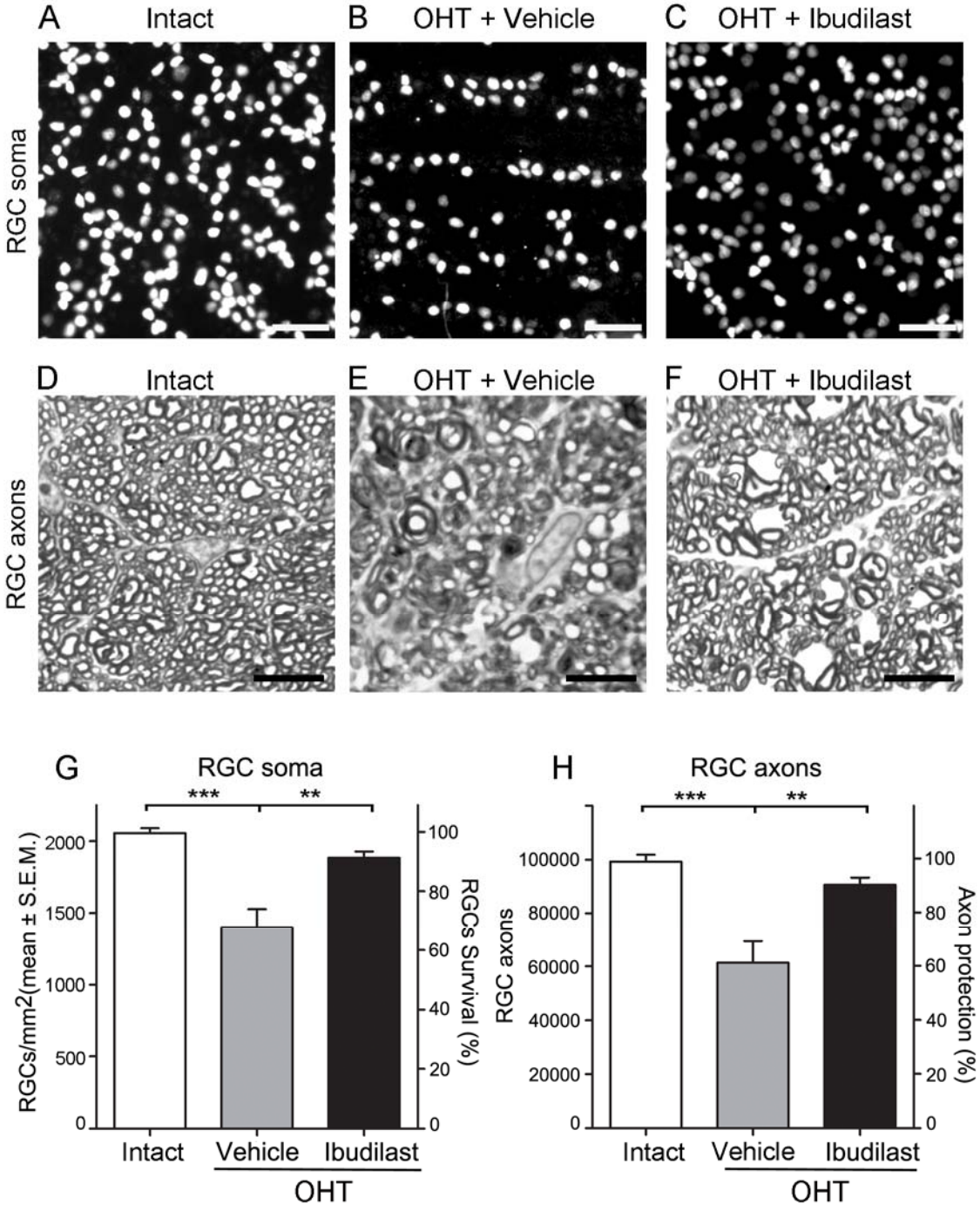

A
Intact
B
OHT + Vehicle
C
OHT + Ibudilast
RGC soma
D
Intact
E
OHT + Vehicle
F
OHT + Ibudilast
RGC axons
G
RGC soma
***
**
RGCs/mm2(mean ± S.E.M.)
RGCs Survival (%)
Intact
Vehicle
Ibudilast
OHT
H
RGC axons
***
**
RGC axons
Axon protection (%)
Intact
Vehicle
Ibudilast
OHT

FIGURE 6

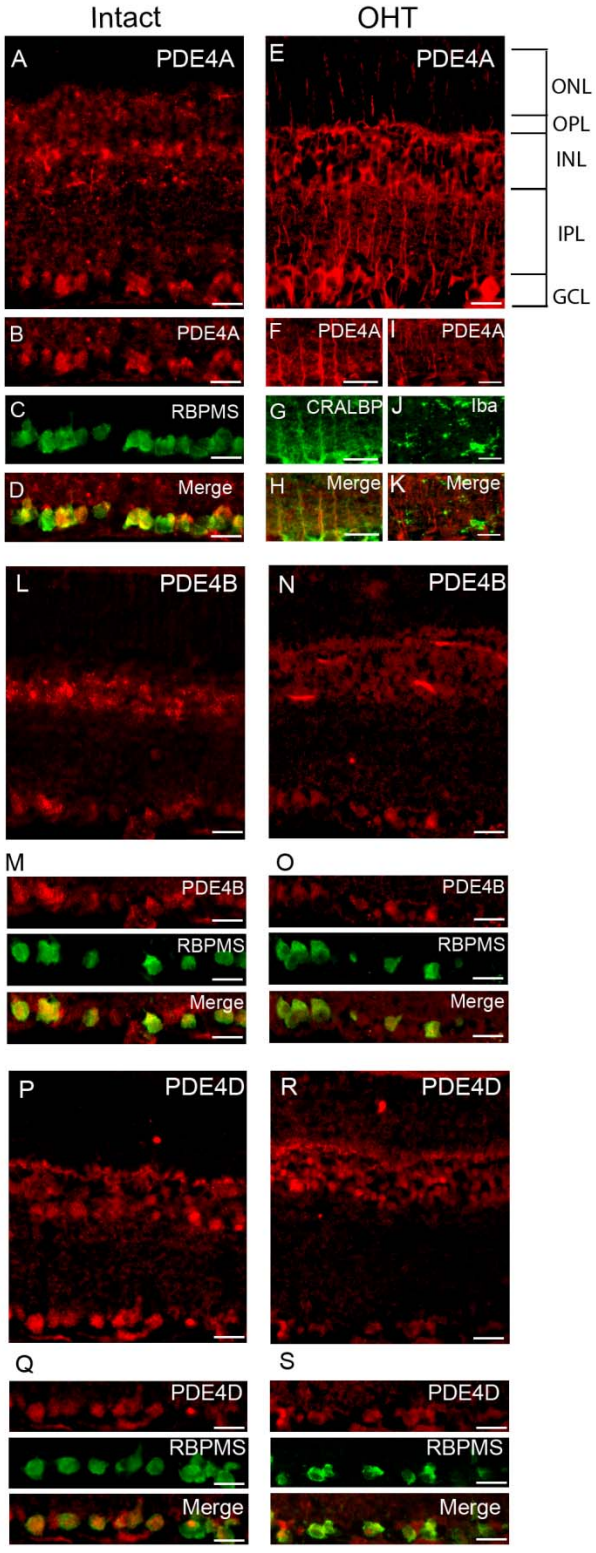

Intact
OHT
A
PDE4A
E
PDE4A
ONL
OPL
INL
IPL
GCL
B
PDE4A
F
PDE4A
I
PDE4A
C
RBPMS
G
CRALBP
J
Iba
D
Merge
H
Merge
K
Merge
L
PDE4B
N
PDE4B
M
PDE4B
RBPMS
Merge
O
PDE4B
RBPMS
Merge
P
PDE4D
R
PDE4D
Q
PDE4D
RBPMS
Merge
S
PDE4D
RBPMS
Merge

FIGURE 7

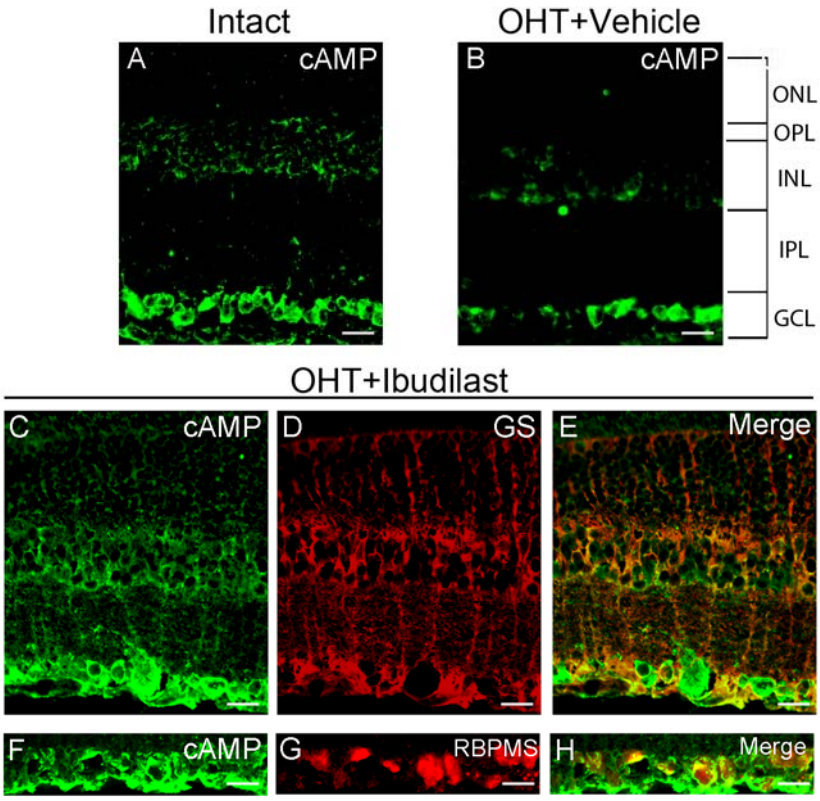
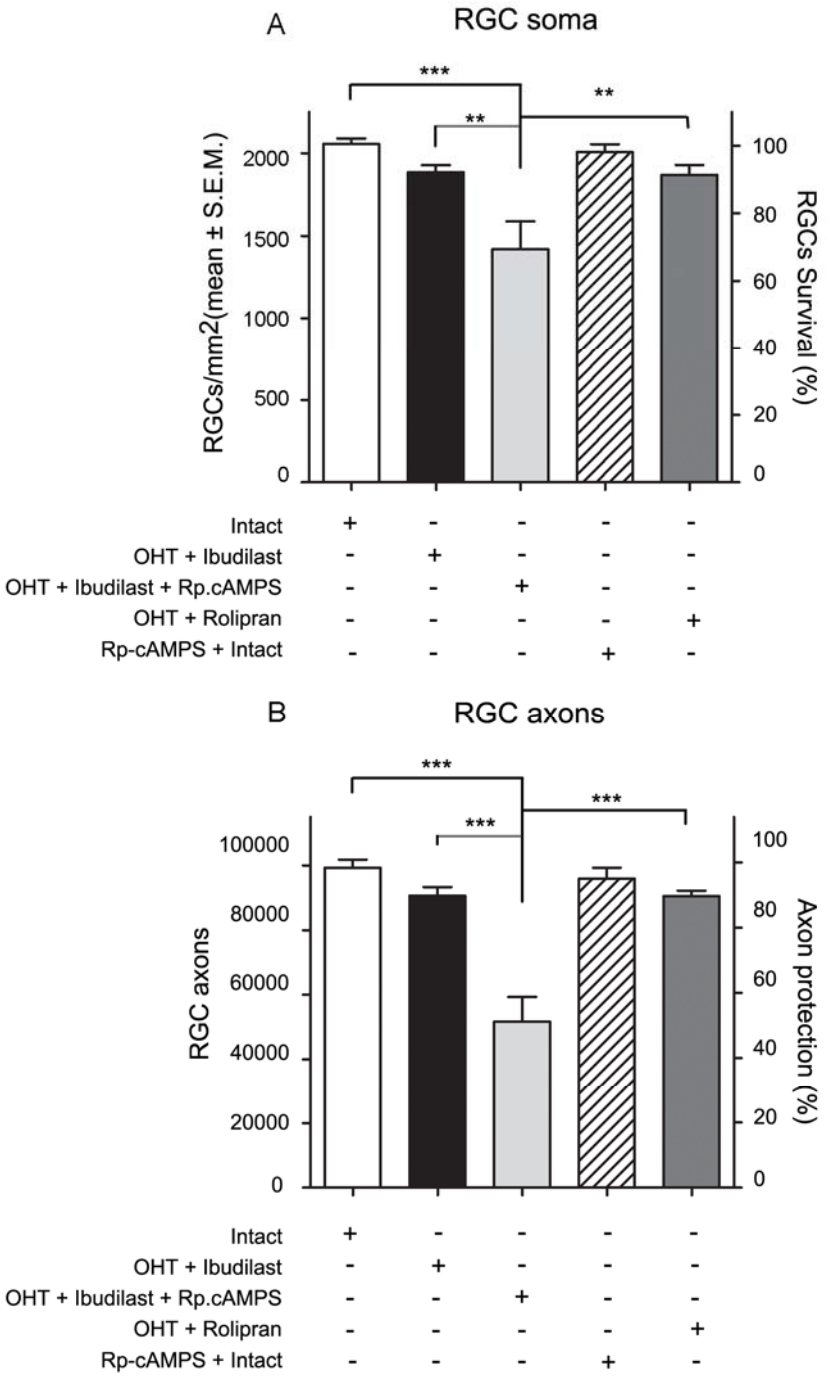

Intact
OHT+Vehicle
A
cAMP
B
cAMP
ONL
OPL
INL
IPL
GCL
OHT+Ibudilast
C
cAMP
D
GS
E
Merge
F
cAMP
G
RBPMS
H
Merge

FIGURE 8


A
RGC soma
***
**
**
RGCs/mm2(mean ± S.E.M.)
2000
1500
1000
500
0
100
80
60
40
20
0
RGCs Survival (%)
Intact + - - - -
OHT + Ibudilast - + - - -
OHT + Ibudilast + Rp.cAMPS - - + - -
OHT + Rolipran - - - - +
Rp-cAMPS + Intact - - - + -
B
RGC axons
***
***
***
RGC axons
100000
80000
60000
40000
20000
0
100
80
60
40
20
0
Axon protection (%)
Intact + - - - -
OHT + Ibudilast - + - - -
OHT + Ibudilast + Rp.cAMPS - - + - -
OHT + Rolipran - - - - +
Rp-cAMPS + Intact - - - + -

CHAPTER 4

IV. GENERAL DISCUSSION

IV.1. Rat model of ocular hypertension (Morrison model)

The studies presented in this thesis were carried out using a well-characterized rat model of ocular hypertension glaucoma. This experimental paradigm is based on scarring of the trabecular meshwork tissue following injection of a hypertonic saline solution (NaCl 1.8 M) which increases the resistance to aqueous humor outflow resulting in gradual elevation of eye pressure and selective loss of RGCs. The ocular hypertension produced by this model is variable and depends on many factors, including the quantity of aqueous humor produced by the animal as well as the degree of obstruction generated by the surgery. Variability is also a normal phenomenon observed in human glaucomatous eyes. Nonetheless, this model displays an excellent linear correlation between intraocular pressure (IOP) and neuronal death with similar pathologies to those observed in human glaucoma (Morrison et al., 1997; Morrison et al., 2005)

IV.2. Glial response and TNFα modulation in experimental glaucoma

Early neuroinflammatory responses mediated by glial cells have been proposed to contribute to the pathogenesis of glaucoma. However, the mechanisms by which glial cells mediate neuronal death in this disease are poorly understood. A better understanding of the progression of the inflammatory response at different stages of the disease is likely to have a significant impact on the identification of novel therapeutic targets for glaucoma.

The first article, presented in chapter 2 of this thesis, is focused on the role of glia-derived soluble TNFα in our rat model of glaucoma. TNFα is one of the most important pro-inflammatory cytokines involved in several physiological and pathological conditions in the CNS. Constitutive levels of TNFα are essential for mediating homeostasis, regulating the

circadian rhythm, glial transmission and synaptic plasticity (Stellwagen, 2011; Santello and Volterra, 2012). However, TNFα appears to be detrimental as high levels of this cytokine have been found in several neurodegenerative diseases, including glaucoma, suggesting a potential role in neuronal death (Yuan and Neufeld, 2000; Sawada et al., 2010). In addition, intravitreal injection of TNFα-induced optic nerve axonal damage as well as RGC death, mimicking the pathology observed in different animal glaucoma models (Nakazawa et al., 2006). Our data showed that induction of glaucoma in rats through the elevation of IOP rapidly stimulates production of TNFα by Müller cells and Iba1-positive microglia/macrophages with macrophage-like morphology. This finding supports previous studies showing that microglia in the glaucomatous rat retina and human optic nerve head express TNFα (Yuan and Neufeld, 2001; Roh et al., 2012). TNFα production is the result of nuclear factor kappa B (NF-κB) activation in Müller cells (Lebrun-Julien et al., 2009) and astrocytes (Tezel et al., 2012) suggesting that NF-κB, the major regulator of inflammatory gene expression, might play a role in the upregulation of glial-derived TNFα in the injured retina. Since TNFα has also been shown to activate NF-κB, it is also possible that TNFα acts directly on glial cells through a positive feedback autocrine loop to enhance their activation and further production of pro-inflammatory cytokines (Kuno et al., 2005).

TNFα signal transduction involves two distinct receptors, TNF receptor 1 (TNFR1) containing a cytoplasmic death domain and TNF receptor 2 (TNFR2) that lacks a functional death domain. These differences contribute to their distinct biological roles. TNFR1, which is expressed in most CNS cell types, has been associated with cell death and inflammation, whereas TNFR2, expressed by some neurons, microglia, and endothelial cells, leads to activation of anti-apoptotic and pro-inflammatory signaling pathways.

The data presented in this thesis indicate that there is a significant increase of TNFR1 mRNA and protein levels in the glaucomatous retina compared to the intact (non-injured) retina. Moreover, immunohistochemical analysis demostrated that TNFR1 increases in RGCs and astrocytes. Our findings are consistent with other studies showing upregulation of TNFR1 in RGCs and the optic nerve head of glaucomatous patients (Yan et al., 2000; Tezel et al., 2001). Furthermore, the progression of optic nerve damage has been associated with the upregulation of TNFR1 (Yuan and Neufeld, 2000). Thus, these results suggest that ocular hypertension-induced upregulation of TNFR1 in RGCs contributes to these cells being highly sensitive to TNFα-mediate damage. Of interest, the upregulation of TNFR1 has also been reported in astrocytes (Tezel et al., 2012). However, the upregulation of TNFR1 in astrocytes does not lead to apoptosis. Indeed, astrocytes appear to be more resistant to apoptosis-inducing signals likely due to the presence of the protein enriched in astrocytes-15 KDa (PEA-15), which inhibits pro-apoptotic TNFR1-elicited signals (Kitsberg et al., 1999; Song et al., 2006).

Our data also demonstrate that TNFR2 mRNA and protein expression is upregulated in the retina early after induction of ocular hypertension. Immunohistochemical analysis confirmed that TNFR2 increases primarily in glaucomatous RGCs. The expression of TNFR2 has been mainly associated to neuronal survival/neuroprotection. For example, retinal TNFR2 is upregulated after ischemia, and TNFR2-deficient mice display exacerbated neuronal death possibly due to defects in PI3K/Akt signaling which have been reported to inhibit the pro-apoptotic proteins (Fontaine et al., 2002). These results suggest a protective role of TNFR2 during retinal ischemia. Conversely, RGC soma and axon protection was observed in TNFR2-deficient mice after induction of ocular hypertension, suggesting that TNFR2 has a toxic effect in this model (Nakazawa et al., 2006). These contradictory results are partially due to

compensatory mechanisms achieved in those animals. To clarify the role of soluble versus trans-membrane TNFα, and their potential impact on TNFR1 or TNFR2 signaling in glaucoma, we utilized XPro1595, a dominant-negative engineered peptide that selectively binds soluble TNFα. Xpro1595 binds with wild-type TNFα monomers and blocks its interaction with TNF receptors without interfering with the transmembrane TNFα. Since soluble TNFα has higher affinity for TNFR1 and trans-membrane TNFα has higher affinity for TNFR2, Xpro1595 is ideal for discerning the specific effect of soluble TNFα on TNFR1 (Steed et al., 2003; Zalevsky et al., 2007). However, due to the differing roles of the two receptors, it would be essential to evaluate the role of each receptor separately. One approach to achieve this evaluation would be the use of small interfering-RNA (siRNA) to directly knock down either TNFR1 or TNFR2 expression. Another possibility would be the use of conditional ablation of TNFRs. Of interest, it has recently been reported that TNFR2 signaling in microglia/macrophages has detrimental effect in EAE, whereas in oligodendrocytes TNFR2 has been associated to promote remyelination in the same model (Gao H, 2014; Madsen P, 2014). These approaches suggest that TNFα exerts different functions, depending on its cell target. Our lab is currently testing AAV containing MiniPromoters, small promoters bioinformatically designed, that exhibit highly specific expression patterns. For instance, knock down TNFα expression in Müller cells would be possible by delivery of siRNA inserted in AAV containing Müller cells-specific MiniPromoter.

IV.3. Protection of RGC soma and axons with XPro1595 in glaucoma

In the study presented in chapter 2, we used XPro1595 due to its advantages for the treatment of chronic neurodegenerative disease like glaucoma. First, XPro1595 is a small synthetic peptide that easily crosses the blood-retinal-barrier. Second, unlike non-selective

inhibitors, including etanercept, infliximab and adalimumab, Xpro1595 preserves the transmembrane TNFα function, maintaining the significant benefits of targeting soluble TNFα. Our data showed that intravitreal injection of XPro1595, and thus, blockade of TNFR1 signaling, in glaucomatous eyes effectively promoted RGC soma protection without affecting the IOP, suggesting that TNFR1-mediated signaling has a toxic effect. Another hallmark of glaucomatous damage is the degeneration of RGC axons in the optic nerve. RGC axons convey the information from the retina to the brain, where they establish synapses with other neurons in the superior colliculus or the lateral geniculate nucleus. In glaucoma, RGC axon degeneration becomes evident due to the presence of irregularities in the axon bundles including loss of their myelin sheaths and axonal swelling (Quigley et al., 1988; Morrison et al., 1997; Chauhan et al., 2002). Our results also showed that blockade of soluble TNFα by intravitreal injection of XPro1595 leads to a robust RGC axons protection suggesting that TNFR1 signaling contributes to RGC axon degeneration.

IV.4. TNFα mediates membrane insertion of CP-AMPARs in RGCs after ocular hypertension induction.

The classical pathway suggests that TNFα/TNFR1 signaling leads to apoptosis by cleaving pro-caspase-8 into caspase-8 (Hsu et al., 1996; Thorburn, 2004). However, previous studies, including our own have shown that TNFα-mediated toxicity involves alternative mechanisms rather than caspase-8 activation (Tezel and Yang, 2004; Lebrun-Julien et al., 2009). Indeed, our data demonstrated that inhibiting caspase-8 activation using z-IETD-fmk did not promote RGC survival in glaucomatous retina (Fig.1). TNFα modulates synaptic transmission and plasticity by its ability to interact with AMPA receptors. Particularly, soluble TNFα strengthens synapses in hippocampal neurons by recruiting AMPA receptors lacking the

subunit GluA2, thus enhancing neuronal Ca^{2+} influx and the response to glutamate (Beattie et al., 2002; Stellwagen et al., 2005). To address the hypothesis that soluble TNFα modulates CP-AMPAR expression in glaucoma, we performed a Co^{2+} permeability assay which is based on the principle that Co^{2+}, like Ca^{2+}, permeates CP-AMPARs with equal ability (Pruss et al., 1991). Importantly, Co^{2+} does not permeate NMDA receptors (Mayer and Westbrook, 1987) or voltage-gated channels (Hagiwara and Byerly, 1981). These properties make the Co^{2+} permeability assay very useful when evaluating the presence of CP-AMPARs in the retina. Our results show that basal expression of CP-AMPARs is restricted to inhibitory cells, horizontal cells, AII and A17 amacrine cells, confirming previous reports (Morkve et al., 2002; Osswald et al., 2007; Diamond, 2011). Interestingly, elevated IOP triggered upregulation of CP-AMPARs in the inner plexiform layer and the RGCs, which was blocked by XPro1595. These results indicate that high IOP increases the level of soluble TNFα expression, which in turn mediates the membrane insertion of CP-AMPARs in RGCs. Increased cell surface insertion of CP-AMPARs triggers Ca^{2+}-dependent RGC death. Indeed, excessive intracellular Ca^{2+} may be taken up by mitochondria resulting in the generation of reactive oxygen species and release of cytochrome c. Ca^{2+} accumulation could also lead to the generation of nitric oxide and the release of mitochondrial apoptosis inducing factor (AIF). In addition, cell death could be triggered by Zn^{2+}-dependent mechanisms as CP-AMPARs are highly permeable to this cation. Zn^{2+} is more potent at inducing mitochondrial disruption (Sensi et al., 2000; Sensi et al., 2003). For example while 10-20 μM of Ca^{2+} is toxic, only 10-100 nM of Zn^{2+} is necessary to produce the same level of neuronal loss. Zn^{2+} toxicity has been reported in oxidative stress and age-related neurodegeneration such as Alzheimer's disease,

where it has been shown to contribute to the aggregation and toxicity of amyloid-β (Sensi et al., 2009).

Of interest, it has been demonstrated in primary microglia the TNFα/TNFR1 complex promotes excitotoxicity by increasing glutamate release in an autocrine manner (Takeuchi et al., 2006). TNFα can also increase the extracellular levels of glutamate by inducing glutamate release from astrocytes through a Ca^{2+}-dependent process mediated by prostaglandins (Bezzi et al., 1998). In the retina, increased levels of glutamate impairment of glutamate metabolism by inhibition of glutamate transporter and glutamine synthetase could lead to the damage of RGCs as well as ONH astrocyte dysfunction (Ju et al., 2014). These studies suggest that glial glutamate release could have detrimental effects in a model of glaucoma, which could be through CP-AMPAR.

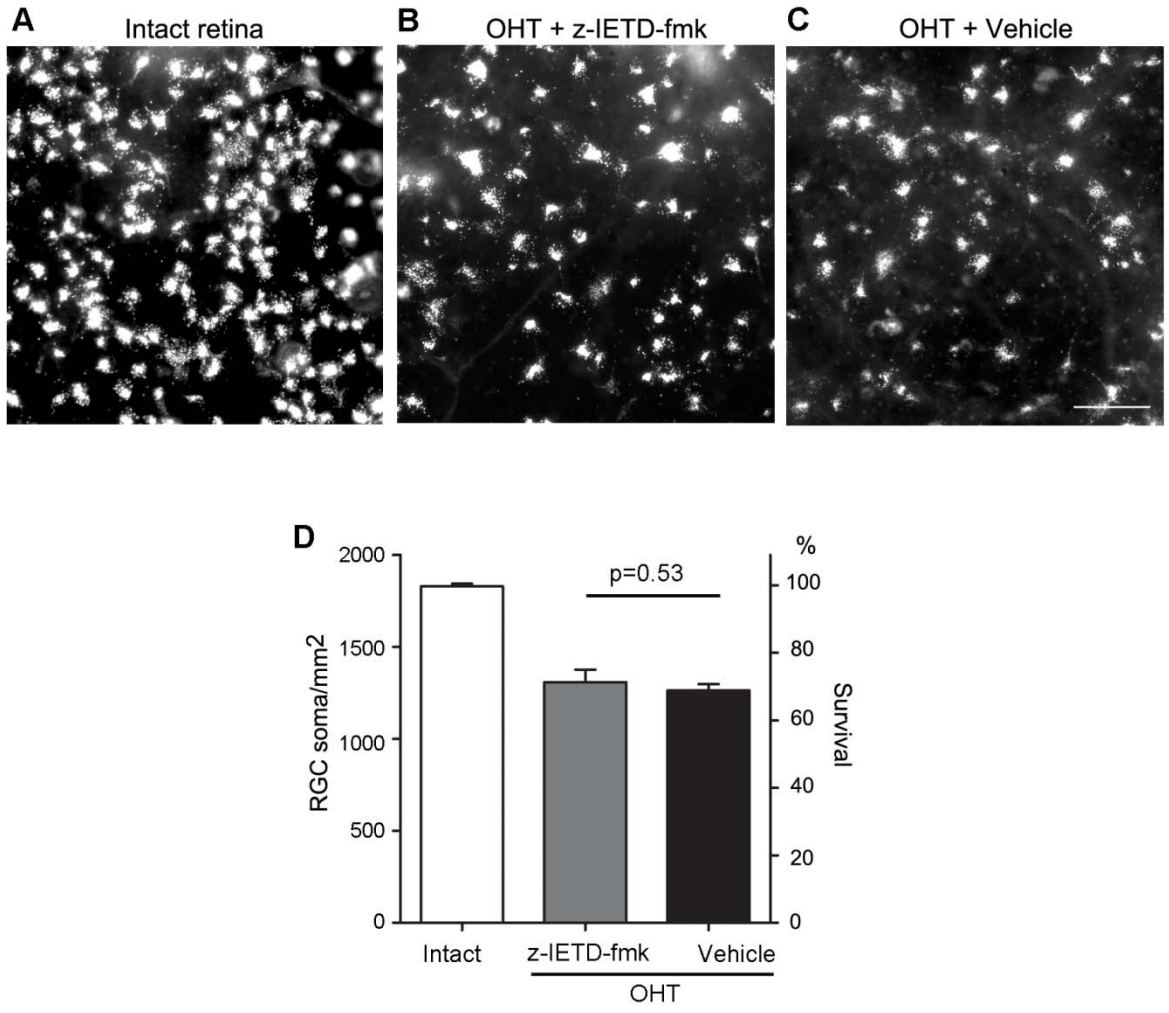


Figure 1. Glial-derived soluble TNFα mediates neuronal loss by mechanisms caspase 8-independent. A-C. Immunohistochemistry analysis of flat-mounted retinas showed higher densities of DiI-labeled RGCs compared to the retinas treated with z-IETD-fmk, a caspase-8 inhibitor, and vehicle. **D.** Quantitative analysis of RGCs confirmed that z-IETD-fmk-treated retina did not promote significant RGC protection in experimental glaucoma. Values are expressed as mean ± S.E.M. (Student's t-test, $p>0.05$). Scale bar: 70 µm.

IV.5. AMPA receptors become CP-AMPARs

Under physiological conditions, AMPA receptors are not Ca^{2+} permeable. However, under certain circumstances they become permeable to divalent ions. Ca^{2+} permeability occurs when there is failure of GluA2 mRNA post-transcriptional editing, where the change from the uncharged amino acid glutamine (Q) to the positively charge arginine (R) in the GluA2 subunit confers permeability to Ca^{2+} and Zn^{2+}. Also, AMPA receptors become permeable to Ca^{2+} when there is decrease in GluA2 expression leading to GluA2-lacking AMPA receptors which is highly permeable to divalent ions.

Our results are consistent with a fully edited GluA2 subunit in retinas with elevated IOP. We performed a nested PCR to isolate a GluA2 254 bp amplicon. This amplicon was not digested with the *Bbvl* restriction enzyme, indicating it did not have the specific restriction site (GCAGC), which is unique to the unedited GluA2 (Q) site. Additionally, the sequence of the 254 bp amplicon confirmed the presence of arginine (R) in the Q/R site of the GluA2 subunit. Contrary to our results, it has been recently suggested that Ca^{2+} permeability is due to the unedited GluA2 in a mouse model of glaucoma (Wang et al., 2014a). This study showed a decrease of ADAR2 expression, the enzyme that converts adenosine to inosine in the double-stranded RNA substrates at six weeks after glaucoma induction compared to the three week time-line of our study. However, a limitation of the Wang et al. study is that a decrease in ADAR2 expression could result from RGC loss at 6 weeks after induction of ocular hypertension. Moreover, the authors did not verify the presence of glutamine (Q) in the Q/R site of the GluA2 subunit as a consequence of elevated IOP. Thus, it remains a possibility that although there is a decrease in ADAR2 expression in response to the induction of glaucoma, this does not necessarily translate to RNA editing of the GluA2 subunit. Another drawback of

this study is that the effect of ADAR2 was only evaluated *in vitro* (Wang et al. 2014), which is often confounded by the culture conditions and does not provide an accurate representation of glaucomatous damage.

Our findings demonstrate that elevated IOP leads to downregulation of GluA2 expression in RGCs. Also, we showed that the blockade of CP-AMPARs using selective antagonists promoted robust RGC soma and axon protection. These results suggest that downregulation of GluA2 in glaucoma is an important mechanism leading to increased Ca^{2+} permeability through AMPA receptors that contributes to the RGC loss. Therefore, maintaining the expression of the GluA2 subunit could potentially be used to protect RGCs. To achieve this, the use of AAV containing the GluA2 gene may be a promising approach. The relative level of neuronal GluA2 expression is not stable, but rather closely regulated by the environment, drugs, seizures and ischemic damage (Pollard et al., 1993; Fitzgerald et al., 1995; Prince et al., 1995; Gorter et al., 1997; Liu and Cull-Candy, 2000). During forebrain ischemia, GluA2 expression is selectively downregulated in CA1 hippocampal neurons destined to die, a process that involves suppression of GluA2 promoter activity by REST (repressor element-1 silencing transcription factor) (Pellegrini-Giampietro et al., 1992; Calderone et al., 2003). A recent study demonstrated that the interaction of GluA2 and NSF (*N*-ethylmaleimide-sensitive fusion protein), an ATPase involved in stabilization of GluA2 at the membrane, is disrupted by Plk2 (Polo-like kinase-2) leading to extensive loss of cell surface GluA2 in primary hippocampal neurons (Evers et al., 2010). TNFα has been shown to preferentially promote exocytosis of GluA2-lacking AMPAR through a PI3K-dependent process downstream of TNFR1, but not TNFR2 (Stellwagen et al., 2005). Although the mechanism by which TNFα downregulates GluA2 expression leading to increased CP-

AMPAR activity in RGCs is currently unknown, it could potentially involve disrupted trafficking, altered gene transcription and/or modification of local protein synthesis (Liu and Savtchouk, 2012).

IV.6. Ibudilast attenuates reactive gliosis in experimental glaucoma

In the second paper presented in Chapter 3 of this thesis, we evaluated the neuroprotective properties of ibudilast in experimental glaucoma. Ibudilast is a non-selective phosphodiesterase (PDE) inhibitor that has been used for more than 20 years in Japan for the treatment of asthma and stroke (Rolan et al., 2009). In addition, the drug was launched for the topical treatment of conjunctivitis (Yokogaki, 2002). Currently, it is being evaluated for the treatment of multiple sclerosis, amyotrophic lateral sclerosis, neuropathic pain, and drug addiction. Ibudilast has many advantages over other compounds because it crosses the blood-brain-barrier, it has a safe pharmacological profile, and, unlike specific PDE4 inhibitors, it can be administered as an ophthalmic or as an oral solution.

As discussed extensively in this thesis, elevated IOP generates several changes in the retina including activation of glial cells. Microglia activation has been associated with RGC neurodegeneration by the release of toxic factors such as TNFα, morphological changes, proliferation, and increased phagocytic activity (Rojas et al., 2014). Consistent with this, our results show that elevated IOP results in higher density of Iba1-positive microglia/macrophages in the retina, optic nerve head and the optic nerve proper. This high density of Iba1-positive cells could be due to a dysregulation of the blood-retinal-barrier and a subsequent infiltration of immune cells (Bringmann et al., 2009). Moreover, circulating monocytes have been reported in the retina and optic nerve of age glaucomatous DBA/2J mice

(Howell et al., 2012). In addition, Iba1-positive cells in glaucomatous retinas also upregulate CD68, a low density lipoprotein and a marker for various cells of monocyte origin with high phagocytic activity. Macroglia cells such astrocytes and Müller cells can also become activated in glaucoma causing direct or indirect damage to neurons. Macroglia activation is characterized by the overexpression of GFAP (Hernandez et al., 2008) and, similar to microglia, by the expression TNFα (Lebrun-Julien et al., 2009; Tezel et al., 2012). Our data confirmed that ocular hypertension leads to upregulation of GFAP, TNFα, and others pro-inflammatory such as IL-6, IL-1β, and MIF. High levels of IL-6 have been proposed to have a protective regenerative effect on axons in glaucomatous eyes (Chidlow et al., 2012). Recently, high levels of IL-1β, mediated by a caspase-1-independent mechanism, have been reported in an acute model of ocular hypertension (Chi et al., 2014). MIF expression has not been reported in glaucomatous retina. Of interest, MIF is required for the microglial activation and production of TNFα, IL-6, IL-1β in a model of EAE (Cox et al., 2013). The role of these cytokines in the pathogenesis of glaucoma remains largely unknown and further research is deserved to unravel their mechanism of action. Intraocular injections of ibudilast attenuated gliosis and the upregulation of the pro-inflammatory cytokines mentioned above in our experimental model of glaucoma. The mechanism proposed by which ibudilast regulates the neuroinflammatory response in glaucoma will be discussed.

IV.7. Intravitreal injection of ibudilast attenuates neuroinflammation in glaucomatous retina.

We found that intravitreal injection of ibudilast exerts a positive effect in protecting RGCs by attenuating gliosis. Ibudilast exerts its function primarily by inhibiting PDE4, the most abundant cAMP-specific PDE in the CNS (Bolger et al., 1994) and in the immune

system (Verghese et al., 1995). In 2009, the group of Cooper and colleagues reported that whereas none of the PDE4 subtypes are expressed by Müller cells, all of them are expressed by RGCs in non-injured retinas (Whitaker and Cooper, 2009). However, they did not evaluate whether or not retinal or optic nerve injury, such as ocular hypertension, modulated the expression of PDE4 subtypes. Intriguingly, our findings demonstrate that elevated IOP leads to the appearance of PDE4A protein, but not PDE4B or PDE4D, in Müller cells. High levels of PDE4 expression have been associated with a decrease of intracellular cAMP, a selective modulator of the pro-inflammatory transcription factor NF-κB. For example, cAMP has been shown to trigger a negative effect on NF-κB (Parry and Mackman, 1997). Our data demonstrated a decrease of cAMP levels after induction of ocular hypertension. These findings suggest that elevated IOP could lead to NF-κB activation in Müller cells. In fact, unpublished data generated by our lab showed that NF-κB becomes activated in Müller cells in a murine model of glaucoma. Furthermore, previous studies have shown that there is activation of NF-κB in Müller cells after intravitreal injection of NMDA (Lebrun-Julien et al., 2009) or TNFα (Mac Nair et al., 2014). Given that PDE activity results in low cAMP levels, we examined whether inhibition of PDE4 by intravitreal injection of ibudilast leads to cAMP accumulation in target cells (Huang et al., 2006). We demonstrate that cAMP expression is upregulated in Müller cells of glaucomatous retina after intravitreal injection of ibudilast. cAMP is a key second messenger that regulates many cellular processes, including inflammatory response (Bourne et al., 1974) neuronal survival and axonal growth (Corredor et al., 2012). Given that cAMP signaling might negatively regulate NF-κB-dependent inflammatory responses, we put forward the idea that ibudilast might attenuate inflammation by increasing cAMP levels in Müller cells. Indeed, increased cAMP through activation of its

target protein kinase A (PKA) modulates gene transcription in favor of an anti-inflammatory response. Of interest, it has been reported that PDE4A selectively interacts with $p75^{NTR}$ leading to cAMP hydrolysis and decreased PKA activation. In fact, $p75^{NTR}$-deficient mice showed increased levels of cAMP after nerve injury (Sachs et al., 2007). $p75^{NTR}$ is exclusively expressed by the Müller cells in the adult retina and it has been associated with TNFα synthesis by these glial cells (Lebrun-Julien et al., 2010). Moreover, $p75^{NTR}$ mRNA upregulation has been reported in experimental glaucoma (Guo et al., 2009). Since $p75^{NTR}$ plays multiples roles in tissue repair, blocking its function would most likely cause unintended and adverse effects on retina. Therefore, targeting $p75^{NTR}$-PDE4A may be a more suitable strategy in glaucoma and thus, preserving other $p75^{NT}$-dependent functions. cAMP regulation is mediated by the interaction of $p75^{NT}$ and the unique C-terminal region of PDE4A. Since this C-terminal is not shared by other PDE4 isoforms, it is an excellent target for inhibiting $p75^{NTR}$-PDE4A interaction. Another possibility is the use of AAV. This is likely the most specific strategy to inhibit the expression and interaction of PDE4A selectively in Müller cells. In this context, knock down PDE4A expression in Müller cells would be possible by delivery of siRNA inserted in AAV containing Müller cells-specific MiniPromoter.

Our results also showed the presence of PDE4A, PDE4B and PDE4D in RGCs, which raise the possibility of additional targets for the action of ibudilast on these neurons. We found a marked increased expression of cAMP in RGCs after ibudilast treatment. However, since activation of NF-κB in RGCs has not been observed in glaucomatous retina, it is plausible that the effect of ibudilast on RGCs differs from that on the Müller cells. Previous studies have shown that elevated cAMP strongly promotes RGC survival. For example, cAMP induced by electrical stimulation leads to RGC protection by a mechanism dependent on PKA activation.

These studies have reported translocation and increased levels of TrkB expression, enhancing neurotropic factor responsiveness (Meyer-Franke et al., 1995; Meyer-Franke et al., 1998). Moreover, in the presence of elevated cAMP levels, several trophic factors had a beneficial effect on RGC survival and axonal growth (Goldberg et al., 2002). Downstream of cAMP, the transcription factor cyclic AMP response element-binding protein (CREB) has been proposed to be a major mediator of the neurotrophin response in neurons (Finkbeiner et al., 1997). Indeed, CREB is a transcription factor that regulates several biological processes, including neurotrophin-mediated neuronal survival (Finkbeiner et al., 1997; Riccio et al., 1999). Therefore, we speculate that CREB activation might be another mechanism by which ibudilast could promote RGC soma and axons survival. Lastly, using Rp-cAMPS, a specific membrane-permeable antagonist of cAMP and PKA inhibitor resistant to hydrolysis by PDEs, we demonstrate that ibudilast-induced RGC survival depends on PKA activation. Importantly, our study demonstrates for the first time that elevated IOP triggers PDE4A protein expression in Muller glia identifying these cells as potential targets for beneficial modulation of neuroinflammation in glaucoma.

The results in the current work are selective for soluble TNFα, and therefore, the use of XPro1595 could be recommended for the treatment of glaucoma due to its positive effects in inhibiting neuroinflammation, whereas inhibition of transmembrane TNFα signaling could have detrimental effects on host defense. In addition, XPro1595 is the only commercially available FDA-approved TNFα-antagonist able to cross the blood-brain-barrier (BBB) and attenuate neuroinflammation and subsequent RGCs death in experimental glaucoma. In contrast, TNFα antagonists including infliximab have been demonstrated to have detrimental effects in patients with neurological diseases due to suppression of the immune system and

inhibition of myelination. On the other hand, ibudilast, a non-selective phosphodiesterase inhibitor that was developed in Japan and marketed there since 1989 to treat asthma and post-stroke complications, is known to attenuate glial cell activation (Rolan et al., 2009). Unlike other selective PDE4 inhibitors, ibudilast appears to be well tolerated at the doses used in the treatment of asthma. Ibudilast also is a FDA-approved drug which is able to cross the BBB (Ledeboer et al., 2006; Rolan et al., 2009). In this thesis, we show that ibudilast is a promising treatment for glaucoma by attenuating gliosis and proinflammatory cytokines including TNFα.

Both studies have clear clinical implications and support compelling rationale for moving toward a clinical trial for glaucoma treatment. I would not recommend the use of both approaches simultaneously to abrogate the consequences of having high ocular hypertension. I would recommend a strategy focused on targeting specifically PDE4 in Müller cells. In fact, inhibition of PDE4 in Müller cells could be involved not only in attenuating glial cell activation and neuroinflammation, but also in promoting neurotrophic factors such as BDNF.

IV.8. GENERAL CONCLUSIONS

A better understanding of the molecular and cellular mechanisms underlying glaucoma pathophysiology is essential for the development of more effective therapeutics to prevent neuronal death and vision loss in this disease. The work presented in this thesis uncovered a new role for glia-derived TNFα signaling and RGC death induced by ocular hypertension. Here, we used a small dominant-negative protein, XPro1595, to neutralize soluble TNFα. The robust neuroprotective of XPro1595 allowed the identification of soluble TNFα as an important mediator of RGC death in glaucoma. Our study revealed that soluble TNFα

stimulates the membrane insertion of CP-AMPARs in RGCs contributing to excitotoxicity in glaucomatous retinas. Collectively, these results establish a new link between neuroinflammation and excitotoxic damage in a well-established pre-clinical model of glaucoma.

Reactive gliosis and neuroinflammation have been proposed to contribute to the pathology of glaucoma. To assess the role of the inflammatory response in this disease, we tested the effect of ibudilast, a phosphodiesterase-4 inhibitor and clinically-approved anti-inflammatory drug. Our data show that ibudilast attenuates gliosis, reduces the expression of pro-inflammatory cytokines, and promotes significant RGC soma and axon protection in experimental glaucoma. We demonstrate that while PDE4A, PDE4B and PDE4D are constitutively expressed by RGCs in intact and glaucomatous retinas, intriguingly, ocular hypertension triggers PDE4A protein expression only in Müller cells. Importantly, our results also showed that ibudilast resulted in an increase in cAMP levels in Müller cells, consistent with its ability to inhibit PDE4. These findings suggest that PDE4A expressed by Müller cells could be a potential target to attenuate gliosis and its detrimental effect on RGCs (Fig. 2).

In conclusion, this thesis demonstrates a tangible neuroinflammatory response that contributes to RGC neurodegeneration in glaucoma. Specifically, I addressed the role of reactive macroglia and microglia, and the contribution of pro-inflammatory cytokines, particularly soluble TNFα. Importantly; I also demonstrated that strategies that reduce glial cell activation and the activity of pro-inflammatory mediators are beneficial for RGC neuroprotection and might have implications for the development of novel therapeutic strategies for the treatment and management of glaucoma.

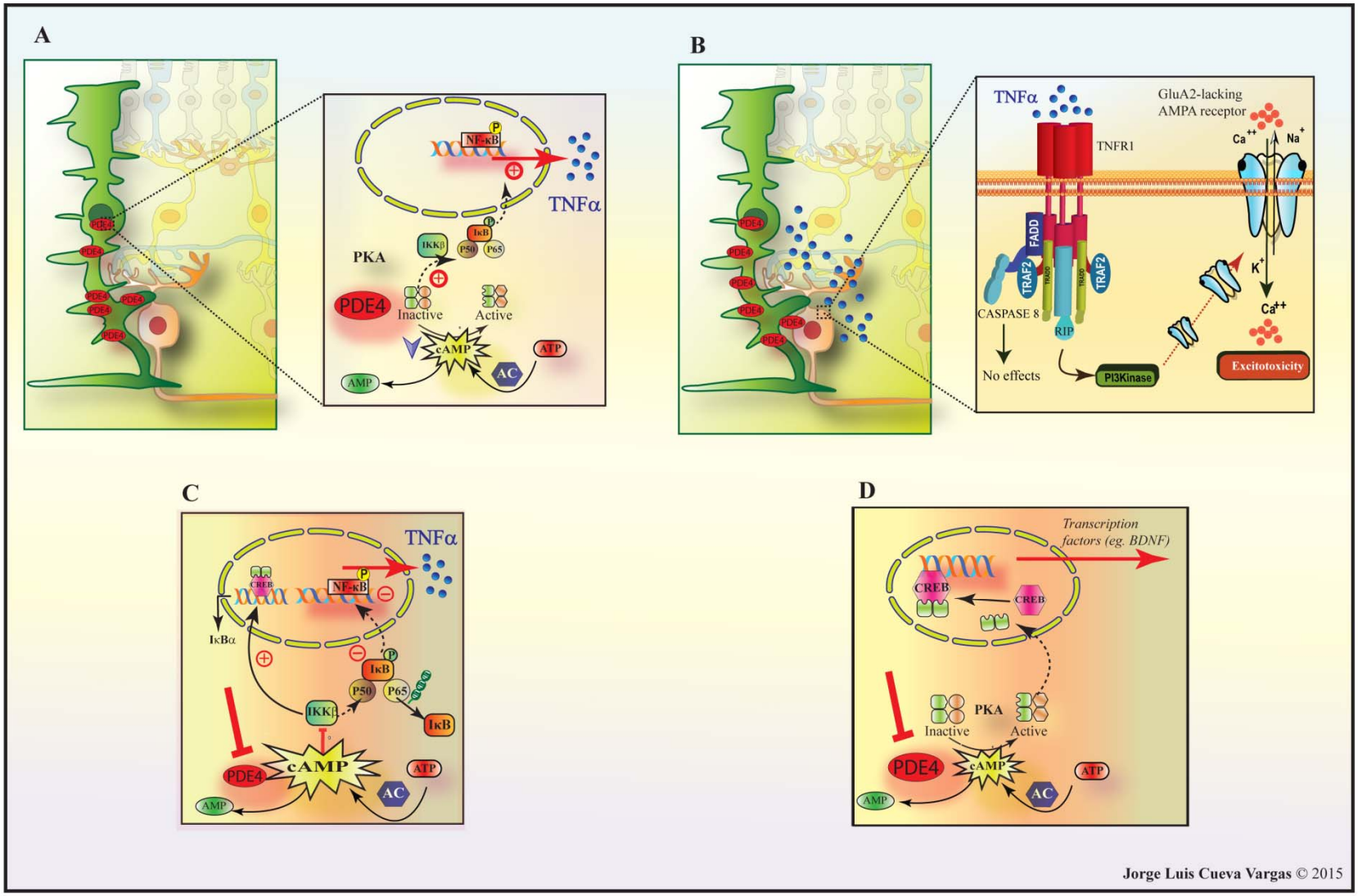

A
B
C
D
TNFα
NF-κB
IKKβ
IκB
P50
P65
PKA
PDE4
Inactive
Active
cAMP
ATP
AMP
AC
GluA2-lacking
AMPA receptor
TNFR1
FADD
TRAF2
TRADD
CASPASE 8
RIP
No effects
PI3Kinase
Excitotoxicity
Ca++
Na+
K+
IκBα
CREB
Transcription
factors (eg. BDNF)
Jorge Luis Cueva Vargas © 2015

Figure 2. Schematic model showing the role of TNFα and PDE4 in experimental glaucoma. A) Elevated IOP leads to substantial expression of PDE4A in Müller cells. The presence of PDE4A decreases cAMP levels which have a positive effect on NF-κB activation and the subsequent expression of TNFα. **B)** TNFα released from Müller cells acts on RCGs mediating the membrane insertion of CP-AMPARs. **C)** Inhibition of PDE4A by administration of ibudilast triggers an increase of cAMP levels in Müller cells. High levels of cAMP have a negative effect on NF-κB activation. **D)** In RGCs, ibudilast treatment leads to activation of PKA. Source: Jorge Luis Cueva Vargas.

V. REFERENCES

Abu-Hassan DW, Li X, Ryan EI, Acott TS, Kelley MJ (2015) Induced pluripotent stem cells restore function in a human cell loss model of open-angle glaucoma. Stem cells 33:751-761.

Adkins JC, Balfour JA (1998) Brimonidine. A review of its pharmacological properties and clinical potential in the management of open-angle glaucoma and ocular hypertension. Drugs & aging 12:225-241.

Allan SM, Tyrrell PJ, Rothwell NJ (2005) Interleukin-1 and neuronal injury. Nature reviews Immunology 5:629-640.

Almasieh M, Zhou Y, Kelly ME, Casanova C, Di Polo A (2010) Structural and functional neuroprotection in glaucoma: role of galantamine-mediated activation of muscarinic acetylcholine receptors. Cell death & disease 1:e27.

Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A (2012) The molecular basis of retinal ganglion cell death in glaucoma. Progress in retinal and eye research 31:152-181.

Anderson DR, Normal Tension Glaucoma S (2003) Collaborative normal tension glaucoma study. Current opinion in ophthalmology 14:86-90.

Anderson MG, Smith RS, Hawes NL, Zabaleta A, Chang B, Wiggs JL, John SW (2002) Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. Nature genetics 30:81-85.

Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell calcium 34:325-337.

Bacher M, Weihe E, Dietzschold B, Meinhardt A, Vedder H, Gemsa D, Bette M (2002) Borna disease virus-induced accumulation of macrophage migration inhibitory factor in rat brain astrocytes is associated with inhibition of macrophage infiltration. Glia 37:291-306.

Bacher M, Meinhardt A, Lan HY, Dhabhar FS, Mu W, Metz CN, Chesney JA, Gemsa D, Donnelly T, Atkins RC, Bucala R (1998) MIF expression in the rat brain: implications for neuronal function. Molecular medicine 4:217-230.

Bacher M, Deuster O, Aljabari B, Egensperger R, Neff F, Jessen F, Popp J, Noelker C, Reese JP, Al-Abed Y, Dodel R (2010) The role of macrophage migration inhibitory factor in Alzheimer's disease. Molecular medicine 16:116-121.

Bai N, Aida T, Yanagisawa M, Katou S, Sakimura K, Mishina M, Tanaka K (2013) NMDA receptor subunits have different roles in NMDA-induced neurotoxicity in the retina. Molecular brain 6:34.

Bai Y, Sivori D, Woo SB, Neet KE, Lerner SF, Saragovi HU (2011) During glaucoma, alpha2-macroglobulin accumulates in aqueous humor and binds to nerve growth factor, neutralizing neuroprotection. Investigative ophthalmology & visual science 52:5260-5265.

Bai Y, Zhu Y, Chen Q, Xu J, Sarunic MV, Saragovi UH, Zhuo Y (2014) Validation of glaucoma-like features in the rat episcleral vein cauterization model. Chinese medical journal 127:359-364.

Balaggan KS, Ali RR (2012) Ocular gene delivery using lentiviral vectors. Gene therapy 19:145-153.

Balaiya S, Edwards J, Tillis T, Khetpal V, Chalam KV (2011) Tumor necrosis factor-alpha (TNF-alpha) levels in aqueous humor of primary open angle glaucoma. Clinical ophthalmology 5:553-556.

Balaratnasingam C, Pham D, Morgan WH, Bass L, Cringle SJ, Yu DY (2009) Mitochondrial cytochrome c oxidase expression in the central nervous system is elevated at sites of pressure gradient elevation but not absolute pressure increase. Journal of neuroscience research 87:2973-2982.

Banerjee D, Dick AD (2004) Blocking CD200-CD200 receptor axis augments NOS-2 expression and aggravates experimental autoimmune uveoretinitis in Lewis rats. Ocular immunology and inflammation 12:115-125.

Baune BT, Wiede F, Braun A, Golledge J, Arolt V, Koerner H (2008) Cognitive dysfunction in mice deficient for TNF- and its receptors. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 147B:1056-1064.

Bayer AU, Ferrari F, Erb C (2002) High occurrence rate of glaucoma among patients with Alzheimer's disease. European neurology 47:165-168.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNFalpha. Science 295:2282-2285.

Bell JD, Park E, Ai J, Baker AJ (2009) PICK1-mediated GluR2 endocytosis contributes to cellular injury after neuronal trauma. Cell death and differentiation 16:1665-1680.

Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:7028-7040.

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, Kooistra T, Fingerle-Rowson G, Ghezzi P, Kleemann R, McColl SR, Bucala R, Hickey MJ, Weber C (2007) MIF is a noncognate ligand of CXC

chemokine receptors in inflammatory and atherogenic cell recruitment. Nature medicine 13:587-596.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 391:281-285.

Blanks JC, Hinton DR, Sadun AA, Miller CA (1989) Retinal ganglion cell degeneration in Alzheimer's disease. Brain research 501:364-372.

Bliss RM, Finckbone VL, Trice J, Strahlendorf H, Strahlendorf J (2011) Tumor necrosis factor-alpha (TNF-alpha) augments AMPA-induced Purkinje neuron toxicity. Brain research 1386:1-14.

Boland MV, Quigley HA (2007) Risk factors and open-angle glaucoma: classification and application. Journal of glaucoma 16:406-418.

Bolger GB, Rodgers L, Riggs M (1994) Differential CNS expression of alternative mRNA isoforms of the mammalian genes encoding cAMP-specific phosphodiesterases. Gene 149:237-244.

Bosco A, Steele MR, Vetter ML (2011) Early microglia activation in a mouse model of chronic glaucoma. The Journal of comparative neurology 519:599-620.

Bosco A, Inman DM, Steele MR, Wu G, Soto I, Marsh-Armstrong N, Hubbard WC, Calkins DJ, Horner PJ, Vetter ML (2008) Reduced retina microglial activation and improved optic nerve integrity with minocycline treatment in the DBA/2J mouse model of glaucoma. Investigative ophthalmology & visual science 49:1437-1446.

Bourne HR, Lichtenstein LM, Melmon KL, Henney CS, Weinstein Y, Shearer GM (1974) Modulation of inflammation and immunity by cyclic AMP. Science 184:19-28.

Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, Skatchkov SN, Osborne NN, Reichenbach A (2006) Muller cells in the healthy and diseased retina. Progress in retinal and eye research 25:397-424.

Bringmann A, Iandiev I, Pannicke T, Wurm A, Hollborn M, Wiedemann P, Osborne NN, Reichenbach A (2009) Cellular signaling and factors involved in Muller cell gliosis: neuroprotective and detrimental effects. Progress in retinal and eye research 28:423-451.

Buie LK, Rasmussen CA, Porterfield EC, Ramgolam VS, Choi VW, Markovic-Plese S, Samulski RJ, Kaufman PL, Borras T (2010) Self-complementary AAV virus (scAAV) safe and long-term gene transfer in the trabecular meshwork of living rats and monkeys. Investigative ophthalmology & visual science 51:236-248.

Bull ND, Johnson TV, Martin KR (2008) Stem cells for neuroprotection in glaucoma. Progress in brain research 173:511-519.

Burnashev N, Khodorova A, Jonas P, Helm PJ, Wisden W, Monyer H, Seeburg PH, Sakmann B (1992) Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science 256:1566-1570.

Calandra T, Bernhagen J, Mitchell RA, Bucala R (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. The Journal of experimental medicine 179:1895-1902.

Calderone A, Jover T, Noh KM, Tanaka H, Yokota H, Lin Y, Grooms SY, Regis R, Bennett MV, Zukin RS (2003) Ischemic insults derepress the gene silencer REST in neurons destined to die. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:2112-2121.

Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R, Lee JC, Cook DN, Jung S, Lira SA, Littman DR, Ransohoff RM (2006) Control of microglial neurotoxicity by the fractalkine receptor. Nature neuroscience 9:917-924.

Carlson T, Kroenke M, Rao P, Lane TE, Segal B (2008) The Th17-ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. The Journal of experimental medicine 205:811-823.

Casson RJ (2006) Possible role of excitotoxicity in the pathogenesis of glaucoma. Clinical & experimental ophthalmology 34:54-63.

Chan WY, Kohsaka S, Rezaie P (2007) The origin and cell lineage of microglia: new concepts. Brain research reviews 53:344-354.

Chauhan BC, Pan J, Archibald ML, LeVatte TL, Kelly ME, Tremblay F (2002) Effect of intraocular pressure on optic disc topography, electroretinography, and axonal loss in a chronic pressure-induced rat model of optic nerve damage. Investigative ophthalmology & visual science 43:2969-2976.

Chen G, Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. Science 296:1634-1635.

Cheng L, Sapieha P, Kittlerova P, Hauswirth WW, Di Polo A (2002) TrkB gene transfer protects retinal ganglion cells from axotomy-induced death in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:3977-3986.

Chi W, Li F, Chen H, Wang Y, Zhu Y, Yang X, Zhu J, Wu F, Ouyang H, Ge J, Weinreb RN, Zhang K, Zhuo Y (2014) Caspase-8 promotes NLRP1/NLRP3 inflammasome activation and IL-1beta production in acute glaucoma. Proceedings of the National Academy of Sciences of the United States of America 111:11181-11186.

Chiasseu M DL, Vande Velde C, Lecrerc N. Di Polo A.. (2015) Thau accumulation in the somato-dendritic compartment of retinal ganglion cells plays a critical role in glaucomatous neurodegeneration. ARVO 2015 Annual Meeting Abstract.

Chidlow G, Ebneter A, Wood JP, Casson RJ (2011) The optic nerve head is the site of axonal transport disruption, axonal cytoskeleton damage and putative axonal regeneration failure in a rat model of glaucoma. Acta neuropathologica 121:737-751.

Chidlow G, Wood JP, Ebneter A, Casson RJ (2012) Interleukin-6 is an efficacious marker of axonal transport disruption during experimental glaucoma and stimulates neuritogenesis in cultured retinal ganglion cells. Neurobiology of disease 48:568-581.

Clem RL, Anggono V, Huganir RL (2010) PICK1 regulates incorporation of calcium-permeable AMPA receptors during cortical synaptic strengthening. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:6360-6366.

Coleman AL, Miglior S (2008) Risk factors for glaucoma onset and progression. Survey of ophthalmology 53 Suppl1:S3-10.

Colton CA, Gilbert DL (1987) Production of superoxide anions by a CNS macrophage, the microglia. FEBS letters 223:284-288.

Conboy L, Varea E, Castro JE, Sakouhi-Ouertatani H, Calandra T, Lashuel HA, Sandi C (2011) Macrophage migration inhibitory factor is critically involved in basal and fluoxetine-stimulated adult hippocampal cell proliferation and in anxiety, depression, and memory-related behaviors. Molecular psychiatry 16:533-547.

Cone FE, Gelman SE, Son JL, Pease ME, Quigley HA (2010) Differential susceptibility to experimental glaucoma among 3 mouse strains using bead and viscoelastic injection. Experimental eye research 91:415-424.

Corredor RG, Trakhtenberg EF, Pita-Thomas W, Jin X, Hu Y, Goldberg JL (2012) Soluble adenylyl cyclase activity is necessary for retinal ganglion cell survival and axon growth. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:7734-7744.

Cox GM, Kithcart AP, Pitt D, Guan Z, Alexander J, Williams JL, Shawler T, Dagia NM, Popovich PG, Satoskar AR, Whitacre CC (2013) Macrophage migration inhibitory factor potentiates autoimmune-mediated neuroinflammation. Journal of immunology 191:1043-1054.

Cuenca N, Fernandez-Sanchez L, Campello L, Maneu V, De la Villa P, Lax P, Pinilla I (2014) Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. Progress in retinal and eye research 43:17-75.

Danesh-Meyer HV (2011) Neuroprotection in glaucoma: recent and future directions. Current opinion in ophthalmology 22:78-86.

Das AV, Mallya KB, Zhao X, Ahmad F, Bhattacharya S, Thoreson WB, Hegde GV, Ahmad I (2006) Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. Developmental biology 299:283-302.

David JR (1966) Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proceedings of the National Academy of Sciences of the United States of America 56:72-77.

Dawson WW, Brooks DE, Hope GM, Samuelson DA, Sherwood MB, Engel HM, Kessler MJ (1993) Primary open angle glaucomas in the rhesus monkey. The British journal of ophthalmology 77:302-310.

Demetriades AM (2011) Gene therapy for glaucoma. Current opinion in ophthalmology 22:73-77.

Depuydt B, van Loo G, Vandenabeele P, Declercq W (2005) Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors. Journal of cell science 118:497-504.

Desai PV, Caprioli J (2008) The treatment of normal-tension glaucoma. Progress in brain research 173:195-210.

Dhingra A, Vardi N (2012) "mGlu Receptors in the Retina" - WIREs Membrane Transport and Signaling. Wiley interdisciplinary reviews Membrane transport and signaling 1:641-653.

Di Polo A, Aigner LJ, Dunn RJ, Bray GM, Aguayo AJ (1998) Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Muller cells temporarily rescues injured retinal ganglion cells. Proceedings of the National Academy of Sciences of the United States of America 95:3978-3983.

Diamond JS (2011) Calcium-permeable AMPA receptors in the retina. Frontiers in molecular neuroscience 4:27.

Dietlein TS, Hermann MM, Jordan JF (2009) The medical and surgical treatment of glaucoma. Deutsches Arzteblatt international 106:597-605; quiz 606.

Dyka FM, May CA, Enz R (2004) Metabotropic glutamate receptors are differentially regulated under elevated intraocular pressure. Journal of neurochemistry 90:190-202.

Eichler W, Yafai Y, Wiedemann P, Reichenbach A (2004) Angiogenesis-related factors derived from retinal glial (Muller) cells in hypoxia. Neuroreport 15:1633-1637.

Emre E, Yuksel N, Duruksu G, Pirhan D, Subasi C, Erman G, Karaoz E (2015) Neuroprotective effects of intravitreally transplanted adipose tissue and bone marrow-derived mesenchymal stem cells in an experimental ocular hypertension model. Cytotherapy.

Erskine L, Herrera E (2014) Connecting the Retina to the Brain. ASN neuro 6.

Erta M, Quintana A, Hidalgo J (2012) Interleukin-6, a major cytokine in the central nervous system. International journal of biological sciences 8:1254-1266.

Esposito E, Cuzzocrea S (2009) TNF-alpha as a therapeutic target in inflammatory diseases, ischemia-reperfusion injury and trauma. Current medicinal chemistry 16:3152-3167.

Eugster HP, Frei K, Bachmann R, Bluethmann H, Lassmann H, Fontana A (1999) Severity of symptoms and demyelination in MOG-induced EAE depends on TNFR1. European journal of immunology 29:626-632.

Euler T, Haverkamp S, Schubert T, Baden T (2014) Retinal bipolar cells: elementary building blocks of vision. Nature reviews Neuroscience 15:507-519.

European Glaucoma Prevention Study G, Miglior S, Pfeiffer N, Torri V, Zeyen T, Cunha-Vaz J, Adamsons I (2007) Predictive factors for open-angle glaucoma among patients with ocular hypertension in the European Glaucoma Prevention Study. Ophthalmology 114:3-9.

Evers DM, Matta JA, Hoe HS, Zarkowsky D, Lee SH, Isaac JT, Pak DT (2010) Plk2 attachment to NSF induces homeostatic removal of GluA2 during chronic overexcitation. Nature neuroscience 13:1199-1207.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031-1047.

Fitzgerald LW, Deutch AY, Gasic G, Heinemann SF, Nestler EJ (1995) Regulation of cortical and subcortical glutamate receptor subunit expression by antipsychotic drugs. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:2453-2461.

Flachsbarth K, Kruszewski K, Jung G, Jankowiak W, Riecken K, Wagenfeld L, Richard G, Fehse B, Bartsch U (2014) Neural stem cell-based intraocular administration of ciliary neurotrophic factor attenuates the loss of axotomized ganglion cells in adult mice. Investigative ophthalmology & visual science 55:7029-7039.

Fogal B, Hewett SJ (2008) Interleukin-1beta: a bridge between inflammation and excitotoxicity? Journal of neurochemistry 106:1-23.

Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U (2002) Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:RC216.

Francke M, Makarov F, Kacza J, Seeger J, Wendt S, Gartner U, Faude F, Wiedemann P, Reichenbach A (2001) Retinal pigment epithelium melanin granules are phagocytozed by Muller glial cells in experimental retinal detachment. Journal of neurocytology 30:131-136.

Franze K, Grosche J, Skatchkov SN, Schinkinger S, Foja C, Schild D, Uckermann O, Travis K, Reichenbach A, Guck J (2007) Muller cells are living optical fibers in the vertebrate retina. Proceedings of the National Academy of Sciences of the United States of America 104:8287-8292.

Gaasterland D, Kupfer C (1974) Experimental glaucoma in the rhesus monkey. Investigative ophthalmology 13:455-457.

Gabelt BT, Kaufman PL (2005) Changes in aqueous humor dynamics with age and glaucoma. Progress in retinal and eye research 24:612-637.

Gallego BI, Salazar JJ, de Hoz R, Rojas B, Ramirez AI, Salinas-Navarro M, Ortin-Martinez A, Valiente-Soriano FJ, Aviles-Trigueros M, Villegas-Perez MP, Vidal-Sanz M, Trivino A, Ramirez JM (2012) IOP induces upregulation of GFAP and MHC-II and microglia reactivity in mice retina contralateral to experimental glaucoma. Journal of neuroinflammation 9:92.

Gao H MP, Brambilla R. (2014) Role of Tumor Necrosis Factor Receptor 2 signaling in microglia and macrophages in experimental autoimmune encephalomyelitis. Society for Neuroscience (SFN) 223.15/H4.

Gerlo S, Kooijman R, Beck IM, Kolmus K, Spooren A, Haegeman G (2011) Cyclic AMP: a selective modulator of NF-kappaB action. Cellular and molecular life sciences : CMLS 68:3823-3841.

Ghosh M, Garcia-Castillo D, Aguirre V, Golshani R, Atkins CM, Bramlett HM, Dietrich WD, Pearse DD (2012) Proinflammatory cytokine regulation of cyclic AMP-phosphodiesterase 4 signaling in microglia in vitro and following CNS injury. Glia 60:1839-1859.

Giangiacomo AaALC (2009) The epidemiology of glaucoma. In: Essentials in Ophthalmology. Glaucoma Progress III (Stamper FGaR, ed): Springer.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330:841-845.

Glabinski AR, O'Bryant S, Selmaj K, Ransohoff RM (2000) CXC chemokine receptors expression during chronic relapsing experimental autoimmune encephalomyelitis. Annals of the New York Academy of Sciences 917:135-144.

Glasgow NG, Siegler Retchless B, Johnson JW (2014) Molecular bases of NMDA receptor subtype-dependent properties. The Journal of physiology.

Goel M, Picciani RG, Lee RK, Bhattacharya SK (2010) Aqueous humor dynamics: a review. The open ophthalmology journal 4:52-59.

Goldberg JH, Tamas G, Aronov D, Yuste R (2003) Calcium microdomains in aspiny dendrites. Neuron 40:807-821.

Goldberg JL, Espinosa JS, Xu Y, Davidson N, Kovacs GT, Barres BA (2002) Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron 33:689-702.

Gordon MO, Beiser JA, Brandt JD, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP, Parrish RK, 2nd, Wilson MR, Kass MA (2002) The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma. Archives of ophthalmology 120:714-720; discussion 829-730.

Gorter JA, Petrozzino JJ, Aronica EM, Rosenbaum DM, Opitz T, Bennett MV, Connor JA, Zukin RS (1997) Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca2+ influx in hippocampal CA1 neurons of gerbil. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:6179-6188.

Greenberg KP, Lee ES, Schaffer DV, Flannery JG (2006) Gene delivery to the retina using lentiviral vectors. Advances in experimental medicine and biology 572:255-266.

Gross RL, Ji J, Chang P, Pennesi ME, Yang Z, Zhang J, Wu SM (2003) A mouse model of elevated intraocular pressure: retina and optic nerve findings. Transactions of the American Ophthalmological Society 101:163-169; discussion 169-171.

Gruol DL (2014) IL-6 Regulation of Synaptic Function in the CNS. Neuropharmacology.

Guo L, Salt TE, Maass A, Luong V, Moss SE, Fitzke FW, Cordeiro MF (2006) Assessment of neuroprotective effects of glutamate modulation on glaucoma-related retinal ganglion cell apoptosis in vivo. Investigative ophthalmology & visual science 47:626-633.

Guo Y, Johnson E, Cepurna W, Jia L, Dyck J, Morrison JC (2009) Does elevated intraocular pressure reduce retinal TRKB-mediated survival signaling in experimental glaucoma? Experimental eye research 89:921-933.

Hagiwara S, Byerly L (1981) Calcium channel. Annual review of neuroscience 4:69-125.

Harada C, Harada T, Quah HM, Maekawa F, Yoshida K, Ohno S, Wada K, Parada LF, Tanaka K (2003) Potential role of glial cell line-derived neurotrophic factor receptors in Muller glial cells during light-induced retinal degeneration. Neuroscience 122:229-235.

Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K (2002) Microglia-Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:9228-9236.

Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K, Saeki T, Aihara M, Yoshida H, Mitani A, Tanaka K (2007) The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. The Journal of clinical investigation 117:1763-1770.

Hare WA, WoldeMussie E, Weinreb RN, Ton H, Ruiz G, Wijono M, Feldmann B, Zangwill L, Wheeler L (2004) Efficacy and safety of memantine treatment for reduction of changes associated with experimental glaucoma in monkey, II: Structural measures. Investigative ophthalmology & visual science 45:2640-2651.

Harvey AR, Hellstrom M, Rodger J (2009) Gene therapy and transplantation in the retinofugal pathway. Progress in brain research 175:151-161.

Harvey AR, Kamphuis W, Eggers R, Symons NA, Blits B, Niclou S, Boer GJ, Verhaagen J (2002) Intravitreal injection of adeno-associated viral vectors results in the transduction of different types of retinal neurons in neonatal and adult rats: a comparison with lentiviral vectors. Molecular and cellular neurosciences 21:141-157.

Hayreh SS, Podhajsky P, Zimmerman MB (1999) Beta-blocker eyedrops and nocturnal arterial hypotension. American journal of ophthalmology 128:301-309.

Helmstaedter M, Briggman KL, Turaga SC, Jain V, Seung HS, Denk W (2013) Connectomic reconstruction of the inner plexiform layer in the mouse retina. Nature 500:168-174.

Hernandez MR, Miao H, Lukas T (2008) Astrocytes in glaucomatous optic neuropathy. Progress in brain research 173:353-373.

Hess A, Axmann R, Rech J, Finzel S, Heindl C, Kreitz S, Sergeeva M, Saake M, Garcia M, Kollias G, Straub RH, Sporns O, Doerfler A, Brune K, Schett G (2011) Blockade of TNF-alpha rapidly inhibits pain responses in the central nervous system. Proceedings of the National Academy of Sciences of the United States of America 108:3731-3736.

Higuchi M, Single FN, Kohler M, Sommer B, Sprengel R, Seeburg PH (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. Cell 75:1361-1370.

Hinton DR, Sadun AA, Blanks JC, Miller CA (1986) Optic-nerve degeneration in Alzheimer's disease. The New England journal of medicine 315:485-487.

Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, Blom B, Homola ME, Streit WJ, Brown MH, Barclay AN, Sedgwick JD (2000) Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 290:1768-1771.

Hoon M, Okawa H, Della Santina L, Wong RO (2014) Functional architecture of the retina: development and disease. Progress in retinal and eye research 42:44-84.

Housley GD, Bringmann A, Reichenbach A (2009) Purinergic signaling in special senses. Trends in neurosciences 32:128-141.

Howell GR, Libby RT, Jakobs TC, Smith RS, Phalan FC, Barter JW, Barbay JM, Marchant JK, Mahesh N, Porciatti V, Whitmore AV, Masland RH, John SW (2007) Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. The Journal of cell biology 179:1523-1537.

Howell GR, Macalinao DG, Sousa GL, Walden M, Soto I, Kneeland SC, Barbay JM, King BL, Marchant JK, Hibbs M, Stevens B, Barres BA, Clark AF, Libby RT, John SW (2011) Molecular clustering identifies complement and endothelin induction as early events in a mouse model of glaucoma. The Journal of clinical investigation 121:1429-1444.

Howell GR, Soto I, Zhu X, Ryan M, Macalinao DG, Sousa GL, Caddle LB, MacNicoll KH, Barbay JM, Porciatti V, Anderson MG, Smith RS, Clark AF, Libby RT, John SW (2012) Radiation treatment inhibits monocyte entry into the optic nerve head and prevents neuronal damage in a mouse model of glaucoma. The Journal of clinical investigation 122:1246-1261.

Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299-308.

Huang W, Fileta J, Rawe I, Qu J, Grosskreutz CL (2010) Calpain activation in experimental glaucoma. Investigative ophthalmology & visual science 51:3049-3054.

Huang Z, Liu S, Zhang L, Salem M, Greig GM, Chan CC, Natsumeda Y, Noguchi K (2006) Preferential inhibition of human phosphodiesterase 4 by ibudilast. Life sciences 78:2663-2668.

Idriss HT, Naismith JH (2000) TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microscopy research and technique 50:184-195.

Inacio AR, Ruscher K, Leng L, Bucala R, Deierborg T (2011) Macrophage migration inhibitory factor promotes cell death and aggravates neurologic deficits after experimental stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 31:1093-1106.

Isenmann S, Schmeer C, Kretz A (2004) How to keep injured CNS neurons viable--strategies for neuroprotection and gene transfer to retinal ganglion cells. Molecular and cellular neurosciences 26:1-16.

Ishikawa M (2013) Abnormalities in glutamate metabolism and excitotoxicity in the retinal diseases. Scientifica 2013:528940.

Jander S, Schroeter M, Stoll G (2000) Role of NMDA receptor signaling in the regulation of inflammatory gene expression after focal brain ischemia. Journal of neuroimmunology 109:181-187.

Jha P, Banda H, Tytarenko R, Bora PS, Bora NS (2011) Complement mediated apoptosis leads to the loss of retinal ganglion cells in animal model of glaucoma. Molecular immunology 48:2151-2158.

Johnson EC, Doser TA, Cepurna WO, Dyck JA, Jia L, Guo Y, Lambert WS, Morrison JC (2011) Cell proliferation and interleukin-6-type cytokine signaling are implicated by gene expression responses in early optic nerve head injury in rat glaucoma. Investigative ophthalmology & visual science 52:504-518.

Johnson TV, Tomarev SI (2010) Rodent models of glaucoma. Brain research bulletin 81:349-358.

Jones RS, Carroll RC, Nawy S (2012) Light-induced plasticity of synaptic AMPA receptor composition in retinal ganglion cells. Neuron 75:467-478.

Ju WK, Kim KY, Noh YH, Hoshijima M, Lukas TJ, Ellisman MH, Weinreb RN, Perkins GA (2014) Increased mitochondrial fission and volume density by blocking glutamate excitotoxicity protect glaucomatous optic nerve head astrocytes. Glia.

Juttler E, Tarabin V, Schwaninger M (2002) Interleukin-6 (IL-6): a possible neuromodulator induced by neuronal activity. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 8:268-275.

Kanamori A, Nakamura M, Nakanishi Y, Yamada Y, Negi A (2005) Long-term glial reactivity in rat retinas ipsilateral and contralateral to experimental glaucoma. Experimental eye research 81:48-56.

Karlstetter M, Scholz R, Rutar M, Wong WT, Provis JM, Langmann T (2014) Retinal microglia: Just bystander or target for therapy? Progress in retinal and eye research.

Kiebala M, Maggirwar SB (2011) Ibudilast, a pharmacologic phosphodiesterase inhibitor, prevents human immunodeficiency virus-1 Tat-mediated activation of microglial cells. PloS one 6:e18633.

Kiel JW, Hollingsworth M, Rao R, Chen M, Reitsamer HA (2011) Ciliary blood flow and aqueous humor production. Progress in retinal and eye research 30:1-17.

Kierdorf K et al. (2013) Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nature neuroscience 16:273-280.

Kimelberg HK (2010) Functions of mature mammalian astrocytes: a current view. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry 16:79-106.

Kitsberg D, Formstecher E, Fauquet M, Kubes M, Cordier J, Canton B, Pan G, Rolli M, Glowinski J, Chneiweiss H (1999) Knock-out of the neural death effector domain protein PEA-15 demonstrates that its expression protects astrocytes from TNFalpha-induced apoptosis. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:8244-8251.

Klein BE, Klein R, Lee KE (2004) Heritability of risk factors for primary open-angle glaucoma: the Beaver Dam Eye Study. Investigative ophthalmology & visual science 45:59-62.

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

Kolb H (1995b) Roles of Amacrine Cells. In: Webvision: The Organization of the Retina and Visual System (Kolb H, Fernandez E, Nelson R, eds). Salt Lake City (UT).

Kortenbruck G, Berger E, Speckmann EJ, Musshoff U (2001) RNA editing at the Q/R site for the glutamate receptor subunits GLUR2, GLUR5, and GLUR6 in hippocampus and temporal cortex from epileptic patients. Neurobiology of disease 8:459-468.

Krueger JM (2008) The role of cytokines in sleep regulation. Current pharmaceutical design 14:3408-3416.

Kuehn MH, Kim CY, Ostojic J, Bellin M, Alward WL, Stone EM, Sakaguchi DS, Grozdanic SD, Kwon YH (2006) Retinal synthesis and deposition of complement components induced by ocular hypertension. Experimental eye research 83:620-628.

Kuno R, Wang J, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A (2005) Autocrine activation of microglia by tumor necrosis factor-alpha. Journal of neuroimmunology 162:89-96.

Kur J, Newman EA, Chan-Ling T (2012) Cellular and physiological mechanisms underlying blood flow regulation in the retina and choroid in health and disease. Progress in retinal and eye research 31:377-406.

Kwak S, Kawahara Y (2005) Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. Journal of molecular medicine 83:110-120.

Lamartina S, Cimino M, Roscilli G, Dammassa E, Lazzaro D, Rota R, Ciliberto G, Toniatti C (2007) Helper-dependent adenovirus for the gene therapy of proliferative retinopathies: stable gene transfer, regulated gene expression and therapeutic efficacy. The journal of gene medicine 9:862-874.

Lambertsen KL, Clausen BH, Babcock AA, Gregersen R, Fenger C, Nielsen HH, Haugaard LS, Wirenfeldt M, Nielsen M, Dagnaes-Hansen F, Bluethmann H, Faergeman NJ, Meldgaard M, Deierborg T, Finsen B (2009) Microglia protect neurons against ischemia

by synthesis of tumor necrosis factor. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:1319-1330.

Lamontagne S, Meadows E, Luk P, Normandin D, Muise E, Boulet L, Pon DJ, Robichaud A, Robertson GS, Metters KM, Nantel F (2001) Localization of phosphodiesterase-4 isoforms in the medulla and nodose ganglion of the squirrel monkey. Brain research 920:84-96.

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

Leaver SG, Cui Q, Plant GW, Arulpragasam A, Hisheh S, Verhaagen J, Harvey AR (2006) AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. Gene therapy 13:1328-1341.

Lebrun-Julien F, Bertrand MJ, De Backer O, Stellwagen D, Morales CR, Di Polo A, Barker PA (2010) ProNGF induces TNFalpha-dependent death of retinal ganglion cells through a p75NTR non-cell-autonomous signaling pathway. Proceedings of the National Academy of Sciences of the United States of America 107:3817-3822.

Lebrun-Julien F, Duplan L, Pernet V, Osswald I, Sapieha P, Bourgeois P, Dickson K, Bowie D, Barker PA, Di Polo A (2009) Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:5536-5545.

Ledeboer A, Liu T, Shumilla JA, Mahoney JH, Vijay S, Gross MI, Vargas JA, Sultzbaugh L, Claypool MD, Sanftner LM, Watkins LR, Johnson KW (2006) The glial modulatory drug AV411 attenuates mechanical allodynia in rat models of neuropathic pain. Neuron glia biology 2:279-291.

Lee HG, Zhu X, O'Neill MJ, Webber K, Casadesus G, Marlatt M, Raina AK, Perry G, Smith MA (2004) The role of metabotropic glutamate receptors in Alzheimer's disease. Acta neurobiologiae experimentalis 64:89-98.

Leibinger M, Muller A, Gobrecht P, Diekmann H, Andreadaki A, Fischer D (2013) Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell death & disease 4:e609.

Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (2003) MIF signal transduction initiated by binding to CD74. The Journal of experimental medicine 197:1467-1476.

Lerma J, Marques JM (2013) Kainate receptors in health and disease. Neuron 80:292-311.

Leske MC, Connell AM, Schachat AP, Hyman L (1994) The Barbados Eye Study. Prevalence of open angle glaucoma. Archives of ophthalmology 112:821-829.

Levkovitch-Verbin H, Kalev-Landoy M, Habot-Wilner Z, Melamed S (2006) Minocycline delays death of retinal ganglion cells in experimental glaucoma and after optic nerve transection. Archives of ophthalmology 124:520-526.

Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME (2002) Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. Investigative ophthalmology & visual science 43:402-410.

Lipsky RH, Marini AM (2007) Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Annals of the New York Academy of Sciences 1122:130-143.

Liu B, Neufeld AH (2000) Expression of nitric oxide synthase-2 (NOS-2) in reactive astrocytes of the human glaucomatous optic nerve head. Glia 30:178-186.

Liu B, Neufeld AH (2004) Activation of epidermal growth factor receptor causes astrocytes to form cribriform structures. Glia 46:153-168.

Liu SJ, Savtchouk I (2012) Ca(2+) permeable AMPA receptors switch allegiances: mechanisms and consequences. The Journal of physiology 590:13-20.

Liu SQ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. Nature 405:454-458.

Liu X, Rasmussen CA, Gabelt BT, Brandt CR, Kaufman PL (2009) Gene therapy targeting glaucoma: where are we? Survey of ophthalmology 54:472-486.

Liu Z, Qiu YH, Li B, Ma SH, Peng YP (2011) Neuroprotection of interleukin-6 against NMDA-induced apoptosis and its signal-transduction mechanisms. Neurotoxicity research 19:484-495.

Llobet A, Gasull X, Gual A (2003) Understanding trabecular meshwork physiology: a key to the control of intraocular pressure? News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society 18:205-209.

London A, Benhar I, Schwartz M (2013) The retina as a window to the brain-from eye research to CNS disorders. Nature reviews Neurology 9:44-53.

Lucas DR, Newhouse JP (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. AMA archives of ophthalmology 58:193-201.

Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacology & therapeutics 109:366-398.

Luna G, Lewis GP, Banna CD, Skalli O, Fisher SK (2010) Expression profiles of nestin and synemin in reactive astrocytes and Muller cells following retinal injury: a comparison with glial fibrillar acidic protein and vimentin. Molecular vision 16:2511-2523.

Mac Nair CE, Fernandes KA, Schlamp CL, Libby RT, Nickells RW (2014) Tumor necrosis factor alpha has an early protective effect on retinal ganglion cells after optic nerve crush. Journal of neuroinflammation 11:194.

Madsen P PS, Gao H, Szymkowski D, Bethea J, Brambilla R. (2014) Oligodendroglial TNFR2 mediates teh protective effects of transmembrane tumor necrosis factor (tmTNF) by promoting remyelination in experimental autoimmune encephalomyelitis. Society for Neuroscience (SFN) 223.10/G11.

Manni G, Oddone F, Parisi V, Tosto A, Centofanti M (2008) Intraocular pressure and central corneal thickness. Progress in brain research 173:25-30.

Martin KR, Levkovitch-Verbin H, Valenta D, Baumrind L, Pease ME, Quigley HA (2002) Retinal glutamate transporter changes in experimental glaucoma and after optic nerve transection in the rat. Investigative ophthalmology & visual science 43:2236-2243.

Martin KR, Quigley HA, Zack DJ, Levkovitch-Verbin H, Kielczewski J, Valenta D, Baumrind L, Pease ME, Klein RL, Hauswirth WW (2003) Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in a rat glaucoma model. Investigative ophthalmology & visual science 44:4357-4365.

Masland RH (2001) The fundamental plan of the retina. Nature neuroscience 4:877-886.

Masland RH (2011) Cell populations of the retina: the Proctor lecture. Investigative ophthalmology & visual science 52:4581-4591.

Masland RH (2012a) The tasks of amacrine cells. Visual neuroscience 29:3-9.

Masland RH (2012b) The neuronal organization of the retina. Neuron 76:266-280.

Matsuda A, Tagawa Y, Yoshida K, Matsuda H, Nishihira J (1997) Expression of macrophage migration inhibitory factor in rat retina and its immunohistochemical localization. Journal of neuroimmunology 77:85-90.

Mayer ML, Westbrook GL (1987) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. The Journal of physiology 394:501-527.

McAlpine FE, Lee JK, Harms AS, Ruhn KA, Blurton-Jones M, Hong J, Das P, Golde TE, LaFerla FM, Oddo S, Blesch A, Tansey MG (2009) Inhibition of soluble TNF signaling in a mouse model of Alzheimer's disease prevents pre-plaque amyloid-associated neuropathology. Neurobiology of disease 34:163-177.

McCoy MK, Martinez TN, Ruhn KA, Szymkowski DE, Smith CG, Botterman BR, Tansey KE, Tansey MG (2006) Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:9365-9375.

McKinnon SJ, Schlamp CL, Nickells RW (2009) Mouse models of retinal ganglion cell death and glaucoma. Experimental eye research 88:816-824.

Mellor IR (2010) The AMPA receptor as a therapeutic target: current perspectives and emerging possibilities. Future medicinal chemistry 2:877-891.

Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15:805-819.

Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, Hanson MG, Jr., Reichardt LF, Barres BA (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21:681-693.

Mi XS, Yuan TF, So KF (2014) The current research status of normal tension glaucoma. Clinical interventions in aging 9:1563-1571.

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181-190.

Mitchell P, Lee AJ, Rochtchina E, Wang JJ (2004) Open-angle glaucoma and systemic hypertension: the blue mountains eye study. Journal of glaucoma 13:319-326.

Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, Bucala R (2002) Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proceedings of the National Academy of Sciences of the United States of America 99:345-350.

Moreno MC, Sande P, Marcos HA, de Zavalia N, Keller Sarmiento MI, Rosenstein RE (2005) Effect of glaucoma on the retinal glutamate/glutamine cycle activity. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19:1161-1162.

Morgan JE (2012) Retina ganglion cell degeneration in glaucoma: an opportunity missed? A review. Clinical & experimental ophthalmology 40:364-368.

Morkve SH, Veruki ML, Hartveit E (2002) Functional characteristics of non-NMDA-type ionotropic glutamate receptor channels in AII amacrine cells in rat retina. The Journal of physiology 542:147-165.

Morrison J, Farrell S, Johnson E, Deppmeier L, Moore CG, Grossmann E (1995) Structure and composition of the rodent lamina cribrosa. Experimental eye research 60:127-135.

Morrison JC, Johnson E, Cepurna WO (2008) Rat models for glaucoma research. Progress in brain research 173:285-301.

Morrison JC, Johnson EC, Cepurna W, Jia L (2005) Understanding mechanisms of pressure-induced optic nerve damage. Progress in retinal and eye research 24:217-240.

Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC (1997) A rat model of chronic pressure-induced optic nerve damage. Experimental eye research 64:85-96.

Moss ML et al. (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 385:733-736.

Nagelhus EA, Horio Y, Inanobe A, Fujita A, Haug FM, Nielsen S, Kurachi Y, Ottersen OP (1999) Immunogold evidence suggests that coupling of K+ siphoning and water transport in rat retinal Muller cells is mediated by a coenrichment of Kir4.1 and AQP4 in specific membrane domains. Glia 26:47-54.

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI (2006) Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:12633-12641.

Namekata K, Harada C, Guo X, Kikushima K, Kimura A, Fuse N, Mitamura Y, Kohyama K, Matsumoto Y, Tanaka K, Harada T (2009) Interleukin-1 attenuates normal tension glaucoma-like retinal degeneration in EAAC1-deficient mice. Neuroscience letters 465:160-164.

Naskar R, Vorwerk CK, Dreyer EB (2000) Concurrent downregulation of a glutamate transporter and receptor in glaucoma. Investigative ophthalmology & visual science 41:1940-1944.

Naude PJ, den Boer JA, Luiten PG, Eisel UL (2011) Tumor necrosis factor receptor cross-talk. The FEBS journal 278:888-898.

Nelson R, Connaughton V (1995) Bipolar Cell Pathways in the Vertebrate Retina. In: Webvision: The Organization of the Retina and Visual System (Kolb H, Fernandez E, Nelson R, eds). Salt Lake City (UT).

Neufeld AH, Hernandez MR, Gonzalez M (1997) Nitric oxide synthase in the human glaucomatous optic nerve head. Archives of ophthalmology 115:497-503.

Neufeld AH, Kawai S, Das S, Vora S, Gachie E, Connor JR, Manning PT (2002) Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. Experimental eye research 75:521-528.

Newman EA (2004) A dialogue between glia and neurons in the retina: modulation of neuronal excitability. Neuron glia biology 1:245-252.

Nguyen JV, Soto I, Kim KY, Bushong EA, Oglesby E, Valiente-Soriano FJ, Yang Z, Davis CH, Bedont JL, Son JL, Wei JO, Buchman VL, Zack DJ, Vidal-Sanz M, Ellisman MH, Marsh-Armstrong N (2011) Myelination transition zone astrocytes are constitutively phagocytic and have synuclein dependent reactivity in glaucoma. Proceedings of the National Academy of Sciences of the United States of America 108:1176-1181.

Nickells RW, Howell GR, Soto I, John SW (2012) Under pressure: cellular and molecular responses during glaucoma, a common neurodegeneration with axonopathy. Annual review of neuroscience 35:153-179.

Nicoletti F, Bruno V, Copani A, Casabona G, Knopfel T (1996) Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? Trends in neurosciences 19:267-271.

Nishikiori N, Osanai M, Chiba H, Kojima T, Mitamura Y, Ohguro H, Sawada N (2007) Glial cell-derived cytokines attenuate the breakdown of vascular integrity in diabetic retinopathy. Diabetes 56:1333-1340.

Nishikura K (2010) Functions and regulation of RNA editing by ADAR deaminases. Annual review of biochemistry 79:321-349.

Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annual review of pharmacology and toxicology 50:295-322.

Nomura A, Shigemoto R, Nakamura Y, Okamoto N, Mizuno N, Nakanishi S (1994) Developmentally regulated postsynaptic localization of a metabotropic glutamate receptor in rat rod bipolar cells. Cell 77:361-369.

Ogata A, Nishihira J, Suzuki T, Nagashima K, Tashiro K (1998) Identification of macrophage migration inhibitory factor mRNA expression in neural cells of the rat brain by in situ hybridization. Neuroscience letters 246:173-177.

Ogoshi F, Yin HZ, Kuppumbatti Y, Song B, Amindari S, Weiss JH (2005) Tumor necrosis-factor-alpha (TNF-alpha) induces rapid insertion of Ca2+-permeable alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate (Ca-A/K) channels in a subset of hippocampal pyramidal neurons. Experimental neurology 193:384-393.

Olmos G, Llado J (2014) Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. Mediators of inflammation 2014:861231.

Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science 164:719-721.

Olveczky BP, Baccus SA, Meister M (2003) Segregation of object and background motion in the retina. Nature 423:401-408.

Onodera S, Suzuki K, Matsuno T, Kaneda K, Takagi M, Nishihira J (1997) Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. Immunology 92:131-137.

Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. Nature reviews Molecular cell biology 4:552-565.

Osborne NN (2008) Pathogenesis of ganglion "cell death" in glaucoma and neuroprotection: focus on ganglion cell axonal mitochondria. Progress in brain research 173:339-352.

Osswald IK, Galan A, Bowie D (2007) Light triggers expression of philanthotoxin-insensitive Ca2+-permeable AMPA receptors in the developing rat retina. The Journal of physiology 582:95-111.

Pahl S, Tapken D, Haering SC, Hollmann M (2014) Trafficking of kainate receptors. Membranes 4:565-595.

Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nature reviews Neuroscience 14:383-400.

Parry GC, Mackman N (1997) Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. Journal of immunology 159:5450-5456.

Pearson VL, Rothwell NJ, Toulmond S (1999) Excitotoxic brain damage in the rat induces interleukin-1beta protein in microglia and astrocytes: correlation with the progression of cell death. Glia 25:311-323.

Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. Trends in neurosciences 20:464-470.

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proceedings of the National Academy of Sciences of the United States of America 89:10499-10503.

Peng PL, Zhong X, Tu W, Soundarapandian MM, Molner P, Zhu D, Lau L, Liu S, Liu F, Lu Y (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. Neuron 49:719-733.

Perigolo-Vicente R, Ritt K, Pereira MR, Torres PM, Paes-de-Carvalho R, Giestal-de-Araujo E (2013) IL-6 treatment increases the survival of retinal ganglion cells in vitro: the role of adenosine A1 receptor. Biochemical and biophysical research communications 430:512-518.

Pinheiro PS, Mulle C (2008) Presynaptic glutamate receptors: physiological functions and mechanisms of action. Nature reviews Neuroscience 9:423-436.

Pollard H, Heron A, Moreau J, Ben-Ari Y, Khrestchatisky M (1993) Alterations of the GluR-B AMPA receptor subunit flip/flop expression in kainate-induced epilepsy and ischemia. Neuroscience 57:545-554.

Popp J, Bacher M, Kolsch H, Noelker C, Deuster O, Dodel R, Jessen F (2009) Macrophage migration inhibitory factor in mild cognitive impairment and Alzheimer's disease. Journal of psychiatric research 43:749-753.

Pow DV, Crook DK (1996) Direct immunocytochemical evidence for the transfer of glutamine from glial cells to neurons: use of specific antibodies directed against the d-stereoisomers of glutamate and glutamine. Neuroscience 70:295-302.

Prince HK, Conn PJ, Blackstone CD, Huganir RL, Levey AI (1995) Down-regulation of AMPA receptor subunit GluR2 in amygdaloid kindling. Journal of neurochemistry 64:462-465.

Prinz M, Priller J (2014) Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. Nature reviews Neuroscience 15:300-312.

Pruss RM, Akeson RL, Racke MM, Wilburn JL (1991) Agonist-activated cobalt uptake identifies divalent cation-permeable kainate receptors on neurons and glial cells. Neuron 7:509-518.

Pulliero A, Seydel A, Camoirano A, Sacca SC, Sandri M, Izzotti A (2014) Oxidative damage and autophagy in the human trabecular meshwork as related with ageing. PloS one 9:e98106.

Qu J, Wang D, Grosskreutz CL (2010) Mechanisms of retinal ganglion cell injury and defense in glaucoma. Experimental eye research 91:48-53.

Quigley HA (2005) New paradigms in the mechanisms and management of glaucoma. Eye 19:1241-1248.

Quigley HA, Addicks EM (1980) Chronic experimental glaucoma in primates. I. Production of elevated intraocular pressure by anterior chamber injection of autologous ghost red blood cells. Investigative ophthalmology & visual science 19:126-136.

Quigley HA, Broman AT (2006) The number of people with glaucoma worldwide in 2010 and 2020. The British journal of ophthalmology 90:262-267.

Quigley HA, Dunkelberger GR, Green WR (1988) Chronic human glaucoma causing selectively greater loss of large optic nerve fibers. Ophthalmology 95:357-363.

Quigley HA, Friedman DS, Congdon NG (2003) Possible mechanisms of primary angle-closure and malignant glaucoma. Journal of glaucoma 12:167-180.

Ransohoff RM, Perry VH (2009) Microglial physiology: unique stimuli, specialized responses. Annual review of immunology 27:119-145.

Razay G, Wilcock GK (2008) Galantamine in Alzheimer's disease. Expert review of neurotherapeutics 8:9-17.

Reichenbach A, Bringmann A (2013) New functions of Muller cells. Glia 61:651-678.

Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358-2361.

Rieke F (2000) Mechanisms of single-photon detection in rod photoreceptors. Methods in enzymology 316:186-202.

Ritch R, Tham CC, Lam DS (2004) Long-term success of argon laser peripheral iridoplasty in the management of plateau iris syndrome. Ophthalmology 111:104-108.

Roh M, Zhang Y, Murakami Y, Thanos A, Lee SC, Vavvas DG, Benowitz LI, Miller JW (2012) Etanercept, a widely used inhibitor of tumor necrosis factor-alpha (TNF-alpha), prevents retinal ganglion cell loss in a rat model of glaucoma. PloS one 7:e40065.

Rojas B, Gallego BI, Ramirez AI, Salazar JJ, de Hoz R, Valiente-Soriano FJ, Aviles-Trigueros M, Villegas-Perez MP, Vidal-Sanz M, Trivino A, Ramirez JM (2014) Microglia in mouse retina contralateral to experimental glaucoma exhibit multiple signs of activation in all retinal layers. Journal of neuroinflammation 11:133.

Rolan P, Hutchinson M, Johnson K (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. Expert opinion on pharmacotherapy 10:2897-2904.

Rosen AM, Stevens B (2010) The role of the classical complement cascade in synapse loss during development and glaucoma. Advances in experimental medicine and biology 703:75-93.

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403:316-321.

Rump A, Sommer C, Gass P, Bele S, Meissner D, Kiessling M (1996) Editing of GluR2 RNA in the gerbil hippocampus after global cerebral ischemia. Journal of cerebral blood flow

and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 16:1362-1365.

Ryskamp DA, Jo AO, Frye AM, Vazquez-Chona F, MacAulay N, Thoreson WB, Krizaj D (2014) Swelling and eicosanoid metabolites differentially gate TRPV4 channels in retinal neurons and glia. The Journal of neuroscience : the official journal of the Society for Neuroscience 34:15689-15700.

Ryskamp DA, Witkovsky P, Barabas P, Huang W, Koehler C, Akimov NP, Lee SH, Chauhan S, Xing W, Renteria RC, Liedtke W, Krizaj D (2011) The polymodal ion channel transient receptor potential vanilloid 4 modulates calcium flux, spiking rate, and apoptosis of mouse retinal ganglion cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:7089-7101.

Sacca SC, Izzotti A (2008) Oxidative stress and glaucoma: injury in the anterior segment of the eye. Progress in brain research 173:385-407.

Sachs BD, Baillie GS, McCall JR, Passino MA, Schachtrup C, Wallace DA, Dunlop AJ, MacKenzie KF, Klussmann E, Lynch MJ, Sikorski SL, Nuriel T, Tsigelny I, Zhang J, Houslay MD, Chao MV, Akassoglou K (2007) p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway. The Journal of cell biology 177:1119-1132.

Samsel PA, Kisiswa L, Erichsen JT, Cross SD, Morgan JE (2011) A novel method for the induction of experimental glaucoma using magnetic microspheres. Investigative ophthalmology & visual science 52:1671-1675.

Sanchez RN, Chan CK, Garg S, Kwong JM, Wong MJ, Sadun AA, Lam TT (2003) Interleukin-6 in retinal ischemia reperfusion injury in rats. Investigative ophthalmology & visual science 44:4006-4011.

Santello M, Volterra A (2012) TNFalpha in synaptic function: switching gears. Trends in neurosciences 35:638-647.

Sapieha PS, Peltier M, Rendahl KG, Manning WC, Di Polo A (2003) Fibroblast growth factor-2 gene delivery stimulates axon growth by adult retinal ganglion cells after acute optic nerve injury. Molecular and cellular neurosciences 24:656-672.

Sappington RM, Calkins DJ (2006) Pressure-induced regulation of IL-6 in retinal glial cells: involvement of the ubiquitin/proteasome pathway and NFkappaB. Investigative ophthalmology & visual science 47:3860-3869.

Sappington RM, Chan M, Calkins DJ (2006) Interleukin-6 protects retinal ganglion cells from pressure-induced death. Investigative ophthalmology & visual science 47:2932-2942.

Sappington RM, Carlson BJ, Crish SD, Calkins DJ (2010) The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. Investigative ophthalmology & visual science 51:207-216.

Sato EA, Ohtake Y, Shinoda K, Mashima Y, Kimura I (2006) Decreased blood flow at neuroretinal rim of optic nerve head corresponds with visual field deficit in eyes with normal tension glaucoma. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 244:795-801.

Sawada A, Neufeld AH (1999) Confirmation of the rat model of chronic, moderately elevated intraocular pressure. Experimental eye research 69:525-531.

Sawada H, Fukuchi T, Tanaka T, Abe H (2010) Tumor necrosis factor-alpha concentrations in the aqueous humor of patients with glaucoma. Investigative ophthalmology & visual science 51:903-906.

Schaal SM, Garg MS, Ghosh M, Lovera L, Lopez M, Patel M, Louro J, Patel S, Tuesta L, Chan WM, Pearse DD (2012) The therapeutic profile of rolipram, PDE target and mechanism of action as a neuroprotectant following spinal cord injury. PloS one 7:e43634.

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011) The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et biophysica acta 1813:878-888.

Schmeer C, Straten G, Kugler S, Gravel C, Bahr M, Isenmann S (2002) Dose-dependent rescue of axotomized rat retinal ganglion cells by adenovirus-mediated expression of glial cell-line derived neurotrophic factor in vivo. The European journal of neuroscience 15:637-643.

Schmidl D, Schmetterer L, Garhofer G, Popa-Cherecheanu A (2015) Pharmacotherapy of Glaucoma. Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics.

Schwartz K, Budenz D (2004) Current management of glaucoma. Current opinion in ophthalmology 15:119-126.

Scuderi GL, Pasquale N (2008) Laser therapies for glaucoma: new frontiers. Progress in brain research 173:225-236.

Seibold LK, SooHoo JR, Kahook MY (2015) Endoscopic cyclophotocoagulation. Middle East African journal of ophthalmology 22:18-24.

Seitz R, Ohlmann A, Tamm ER (2013) The role of Muller glia and microglia in glaucoma. Cell and tissue research 353:339-345.

Sensi SL, Yin HZ, Weiss JH (2000) AMPA/kainate receptor-triggered Zn2+ entry into cortical neurons induces mitochondrial Zn2+ uptake and persistent mitochondrial dysfunction. The European journal of neuroscience 12:3813-3818.

Sensi SL, Paoletti P, Bush AI, Sekler I (2009) Zinc in the physiology and pathology of the CNS. Nature reviews Neuroscience 10:780-791.

Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH (2003) Modulation of mitochondrial function by endogenous Zn2+ pools. Proceedings of the National Academy of Sciences of the United States of America 100:6157-6162.

Shaftel SS, Griffin WS, O'Banion MK (2008) The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. Journal of neuroinflammation 5:7.

Shimazawa M, Ito Y, Inokuchi Y, Yamanaka H, Nakanishi T, Hayashi T, Ji B, Higuchi M, Suhara T, Imamura K, Araie M, Watanabe Y, Onoe H, Hara H (2012) An alteration in the lateral geniculate nucleus of experimental glaucoma monkeys: in vivo positron emission tomography imaging of glial activation. PloS one 7:e30526.

Socodato R, Santiago FN, Portugal CC, Domingues AF, Santiago AR, Relvas JB, Ambrosio AF, Paes-de-Carvalho R (2012) Calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors trigger neuronal nitric-oxide synthase activation to promote nerve cell death in an Src kinase-dependent fashion. The Journal of biological chemistry 287:38680-38694.

Song JH, Bellail A, Tse MC, Yong VW, Hao C (2006) Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:3299-3308.

Soto I, Howell GR (2014) The complex role of neuroinflammation in glaucoma. Cold Spring Harbor perspectives in medicine 4.

Stasi K, Nagel D, Yang X, Wang RF, Ren L, Podos SM, Mittag T, Danias J (2006) Complement component 1Q (C1Q) upregulation in retina of murine, primate, and human glaucomatous eyes. Investigative ophthalmology & visual science 47:1024-1029.

Steed PM et al. (2003) Inactivation of TNF signaling by rationally designed dominant-negative TNF variants. Science 301:1895-1898.

Stellwagen D (2011) The contribution of TNFalpha to synaptic plasticity and nervous system function. Advances in experimental medicine and biology 691:541-557.

Stellwagen D, Beattie EC, Seo JY, Malenka RC (2005) Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:3219-3228.

Stephan AH, Barres BA, Stevens B (2012) The complement system: an unexpected role in synaptic pruning during development and disease. Annual review of neuroscience 35:369-389.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA (2007) The classical complement cascade mediates CNS synapse elimination. Cell 131:1164-1178.

Stieger K, Colle MA, Dubreil L, Mendes-Madeira A, Weber M, Le Meur G, Deschamps JY, Provost N, Nivard D, Cherel Y, Moullier P, Rolling F (2008) Subretinal delivery of recombinant AAV serotype 8 vector in dogs results in gene transfer to neurons in the brain. Molecular therapy : the journal of the American Society of Gene Therapy 16:916-923.

Sun Y, Williams A, Waisbourd M, Iacovitti L, Katz LJ (2015) Stem cell therapy for glaucoma: Science or snake oil? Survey of ophthalmology 60:93-105.

Szydlowska K, Tymianski M (2010) Calcium, ischemia and excitotoxicity. Cell calcium 47:122-129.

Takai Y, Tanito M, Ohira A (2012) Multiplex cytokine analysis of aqueous humor in eyes with primary open-angle glaucoma, exfoliation glaucoma, and cataract. Investigative ophthalmology & visual science 53:241-247.

Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, Sonobe Y, Mizuno T, Suzumura A (2006) Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. The Journal of biological chemistry 281:21362-21368.

Tarongoy P, Ho CL, Walton DS (2009) Angle-closure glaucoma: the role of the lens in the pathogenesis, prevention, and treatment. Survey of ophthalmology 54:211-225.

Tartaglia LA, Goeddel DV (1992) Two TNF receptors. Immunology today 13:151-153.

Taylor DL, Jones F, Kubota ES, Pocock JM (2005) Stimulation of microglial metabotropic glutamate receptor mGlu2 triggers tumor necrosis factor alpha-induced neurotoxicity in concert with microglial-derived Fas ligand. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:2952-2964.

Taylor S, Calder CJ, Albon J, Erichsen JT, Boulton ME, Morgan JE (2011) Involvement of the CD200 receptor complex in microglia activation in experimental glaucoma. Experimental eye research 92:338-343.

Tezel G, Wax MB (2000) Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in

cocultured retinal ganglion cells. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:8693-8700.

Tezel G, Yang X (2004) Caspase-independent component of retinal ganglion cell death, in vitro. Investigative ophthalmology & visual science 45:4049-4059.

Tezel G, Li LY, Patil RV, Wax MB (2001) TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. Investigative ophthalmology & visual science 42:1787-1794.

Tezel G, Yang X, Yang J, Wax MB (2004) Role of tumor necrosis factor receptor-1 in the death of retinal ganglion cells following optic nerve crush injury in mice. Brain research 996:202-212.

Tezel G, Yang X, Luo C, Cai J, Powell DW (2012) An astrocyte-specific proteomic approach to inflammatory responses in experimental rat glaucoma. Investigative ophthalmology & visual science 53:4220-4233.

Tham YC, Li X, Wong TY, Quigley HA, Aung T, Cheng CY (2014) Global Prevalence of Glaucoma and Projections of Glaucoma Burden through 2040: A Systematic Review and Meta-Analysis. Ophthalmology 121:2081-2090.

Thorburn A (2004) Death receptor-induced cell killing. Cellular signalling 16:139-144.

Thoreson WB, Witkovsky P (1999) Glutamate receptors and circuits in the vertebrate retina. Progress in retinal and eye research 18:765-810.

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356:768-774.

Tilleux S, Hermans E (2007) Neuroinflammation and regulation of glial glutamate uptake in neurological disorders. Journal of neuroscience research 85:2059-2070.

van der Zypen E (1977) Experimental morphological study on structure and function of the filtration angel of the rat eye. Ophthalmologica Journal international d'ophtalmologie International journal of ophthalmology Zeitschrift fur Augenheilkunde 174:285-298.

Verghese MW, McConnell RT, Lenhard JM, Hamacher L, Jin SL (1995) Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. Molecular pharmacology 47:1164-1171.

Vetrugno M, Cantatore F, Ruggeri G, Ferreri P, Montepara A, Quinto A, Sborgia C (2008) Primary open angle glaucoma: an overview on medical therapy. Progress in brain research 173:181-193.

Vezzani A, Viviani B (2014) Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology.

Vezzani A, Balosso S, Ravizza T (2008) The role of cytokines in the pathophysiology of epilepsy. Brain, behavior, and immunity 22:797-803.

Vidal L, Diaz F, Villena A, Moreno M, Campos JG, de Vargas IP (2006) Nitric oxide synthase in retina and optic nerve head of rat with increased intraocular pressure and effect of timolol. Brain research bulletin 70:406-413.

Viviani B, Boraso M, Marchetti N, Marinovich M (2014) Perspectives on neuroinflammation and excitotoxicity: a neurotoxic conspiracy? Neurotoxicology 43:10-20.

Viviani B, Bartesaghi S, Gardoni F, Vezzani A, Behrens MM, Bartfai T, Binaglia M, Corsini E, Di Luca M, Galli CL, Marinovich M (2003) Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:8692-8700.

Wang AL, Carroll RC, Nawy S (2014a) Down-regulation of the RNA editing enzyme ADAR2 contributes to RGC death in a mouse model of glaucoma. PloS one 9:e91288.

Wang C, Yang XM, Zhuo YY, Zhou H, Lin HB, Cheng YF, Xu JP, Zhang HT (2012) The phosphodiesterase-4 inhibitor rolipram reverses Abeta-induced cognitive impairment and neuroinflammatory and apoptotic responses in rats. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 15:749-766.

Wang J, Lin J, Schlotterer A, Wu L, Fleming T, Busch S, Dietrich N, Hammes HP (2014b) CD74 indicates microglial activation in experimental diabetic retinopathy and exogenous methylglyoxal mimics the response in normoglycemic retina. Acta diabetologica 51:813-821.

Wassle H (2004) Parallel processing in the mammalian retina. Nature reviews Neuroscience 5:747-757.

Watanabe T, Raff MC (1988) Retinal astrocytes are immigrants from the optic nerve. Nature 332:834-837.

Wax MB, Barrett DA, Pestronk A (1994) Increased incidence of paraproteinemia and autoantibodies in patients with normal-pressure glaucoma. American journal of ophthalmology 117:561-568.

Wax MB, Tezel G, Saito I, Gupta RS, Harley JB, Li Z, Romano C (1998) Anti-Ro/SS-A positivity and heat shock protein antibodies in patients with normal-pressure glaucoma. American journal of ophthalmology 125:145-157.

Weber AJ, Zelenak D (2001) Experimental glaucoma in the primate induced by latex microspheres. Journal of neuroscience methods 111:39-48.

Weinreb RN, Aung T, Medeiros FA (2014) The pathophysiology and treatment of glaucoma: a review. Jama 311:1901-1911.

Weise J, Isenmann S, Klocker N, Kugler S, Hirsch S, Gravel C, Bahr M (2000) Adenovirus-mediated expression of ciliary neurotrophic factor (CNTF) rescues axotomized rat retinal ganglion cells but does not support axonal regeneration in vivo. Neurobiology of disease 7:212-223.

Weiss JH, Sensi SL (2000) Ca2+-Zn2+ permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. Trends in neurosciences 23:365-371.

Whitaker CM, Cooper NG (2009) The novel distribution of phosphodiesterase-4 subtypes within the rat retina. Neuroscience 163:1277-1291.

Wise JB, Witter SL (1979) Argon laser therapy for open-angle glaucoma. A pilot study. Archives of ophthalmology 97:319-322.

Wohl SG, Schmeer CW, Witte OW, Isenmann S (2010) Proliferative response of microglia and macrophages in the adult mouse eye after optic nerve lesion. Investigative ophthalmology & visual science 51:2686-2696.

WoldeMussie E, Ruiz G, Wijono M, Wheeler LA (2001) Neuroprotection of retinal ganglion cells by brimonidine in rats with laser-induced chronic ocular hypertension. Investigative ophthalmology & visual science 42:2849-2855.

Wordinger RJ, Clark AF (1999) Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. Progress in retinal and eye research 18:629-667.

Wright GJ, Puklavec MJ, Willis AC, Hoek RM, Sedgwick JD, Brown MH, Barclay AN (2000) Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. Immunity 13:233-242.

Xiang Y, Li B, Wang JM, Li GG, Zhang H, Manyande A, Tian XB (2014) Gene transfer to human trabecular meshwork cells in vitro and ex vivo using HIV-based lentivirus. International journal of ophthalmology 7:924-929.

Xu L, Wang Y, Wang S, Wang Y, Jonas JB (2007) High myopia and glaucoma susceptibility the Beijing Eye Study. Ophthalmology 114:216-220.

Yan X, Tezel G, Wax MB, Edward DP (2000) Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head. Archives of ophthalmology 118:666-673.

Yokogaki S, Susuki, Y., Ohashi, M. (2002) Introduction of a New Anti-allergic Ophthalmic Solution: Ibudilast Ophthalmic Solution (Ketas Eye drops). Japanese Journal of Ocular Pharmacology 16:24-28.

Yoneda S, Tanihara H, Kido N, Honda Y, Goto W, Hara H, Miyawaki N (2001) Interleukin-1beta mediates ischemic injury in the rat retina. Experimental eye research 73:661-667.

Yu DY, Cringle SJ, Balaratnasingam C, Morgan WH, Yu PK, Su EN (2013) Retinal ganglion cells: Energetics, compartmentation, axonal transport, cytoskeletons and vulnerability. Progress in retinal and eye research 36:217-246.

Yuan L, Neufeld AH (2000) Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. Glia 32:42-50.

Yuan L, Neufeld AH (2001) Activated microglia in the human glaucomatous optic nerve head. Journal of neuroscience research 64:523-532.

Yucel YH, Gupta N, Zhang Q, Mizisin AP, Kalichman MW, Weinreb RN (2006) Memantine protects neurons from shrinkage in the lateral geniculate nucleus in experimental glaucoma. Archives of ophthalmology 124:217-225.

Yun H, Lathrop KL, Yang E, Sun M, Kagemann L, Fu V, Stolz DB, Schuman JS, Du Y (2014) A laser-induced mouse model with long-term intraocular pressure elevation. PloS one 9:e107446.

Zalevsky J, Secher T, Ezhevsky SA, Janot L, Steed PM, O'Brien C, Eivazi A, Kung J, Nguyen DH, Doberstein SK, Erard F, Ryffel B, Szymkowski DE (2007) Dominant-negative inhibitors of soluble TNF attenuate experimental arthritis without suppressing innate immunity to infection. Journal of immunology 179:1872-1883.

Zhang Y, Stone J (1997) Role of astrocytes in the control of developing retinal vessels. Investigative ophthalmology & visual science 38:1653-1666.

Zito KaS, V (2009) NMDA receptor function and physiological modulation. In: Encyclopedia of Neuroscience (Squire L, ed): Oxford: Academic Press.

APPENDIX A

CONTRIBUTION TO THE ARTICLES

For the article "**Soluble tumor necrosis factor alpha promotes retinal ganglion cell death in glaucoma via calcium-permeable AMPA receptor activation**" (Chapter 2 in this thesis), I played a key role in the acquisition, analysis and interpretation of the data. My supervisor was responsible for the conception of the study in collaboration with Dr. Derek Bowie and Dr. Philip Barker. We wrote the article together. The study was principally performed on Brown Norway rats. I performed all the rat surgeries (retrograde Fluorogold and DiI labelling, intraocular injections, ocular hypertension surgeries) as well as the immunohistochemistry experiments, western blotting and RGC survival and quantification on rat retinas as well as axons counting on optic nerves. The laboratory of Dr. Philip Barker provided the reverse transcription PCR results, representing 4 of the 74 panels comprising the 7 figures of this paper. The laboratory of Dr. Derek Bowie performed the cobalt staining results, and the nested PCR representing 11 of the 74 panels of the article. I provided the data for 59 of the 74 panels of the article, and on the 15 remaining panels I performed the glaucoma model. I performed all the statistical analyses and worked alongside my supervisor on the data interpretation.

For the article **"The glial cell modulator ibudilast attenuates the neuroinflammatory response and enhances neuronal viability in glaucoma"** (Chapter 3 in this thesis), Nicolas Belforte and I played a key role in the acquisition, analysis and interpretation of the data. We were responsible for the conception of the study. We wrote the article together. Nicolas and I performed all the animal surgeries as well as the immunohistochemistries, westerns and survival quantifications. Nicolas and I performed all the statistical analysis and worked alongside my supervisor on the data interpretation.