Human Organotypic Retinal Cultures as a model of human retinal ganglion cell degeneration in glaucoma. Effects of epigenetic regulation and mesenchymal stem cell derived growth factors.

A thesis Presented for the Degree of Doctor of Medicine
At the University of East Anglia, Norwich, UK

By

Marina Hopes

School of Medicine

University of East Anglia

October 2014

Abstract

Purpose: Human Organotypic Retinal Cultures (HORCs) have been shown to be a useful experimental model to investigate retinal ganglion cell (RGC) fate in short term models of glaucomatous stress. The aim of the current work was to investigate the long-term fate of RGCs in HORCs and to develop culture conditions to promote the RGC survival. The potential neurotrophic effect of mesenchymal stem cell derived growth factors on the RGC survival was studied and the role of epigenetic regulation of retinal cell gene expression was examined.

Methods: Quantitative real-time polymerase chain reaction (QRT-PCR) was used for assessment of retinal cell marker genes expression, immunohistochemistry and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) for quantitative assessment of apoptotic RGCs. LDH activity in culture media was used to assess total cell death in HORCs.

Results: Serum-free (SF) DMEM/HamF12 with no added antibiotics was found to be the medium of choice for the human retina culture. A statistically significant loss of NeuN-labelled RGCs was documented after 2 weeks in culture using SF DMEM/Ham F12, whereas with Neurobasal medium, the loss was detected after week 1. The numbers of apoptotic RGCs were high under all culture conditions after 1 week. Vascular endothelial growth factor (VEGF) and platelet derived growth factors (PDGFs) conferred a protective effect on RGC survival in long-term HORCs, whereas leukaemia inhibitory factor (LIF) failed to exert this effect. The loss of RGC-derived gene markers expression was selectively altered by the histone deacetylase inhibitor (HDACI) trichostatin A (TSA).

Conclusions: The timing for long-term HORCs use *ex vivo* is dependent on culture conditions. Long-term HORCs can be used for up to 2 weeks in order to prevent detectable RGC loss. Both VEGF and PDGF possess an ability to prolong RGC survival in long-term HORCs. HDAC inhibitor TSA selectively reverses the down-regulation of RGC-derived gene markers expression.

List of Contents

CHAPTER 2

CHAPTER 3

List of figures and tables

CHAPTER 4

CHAPTER 5

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproponic acid
Apaf-1	apoptotic protease-activating factor 1
ATP	adenosine 5'-triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl2	B-cell lymphoma protein
BDNF	brain-derived neurotrophic factor
bmMSC	bone marrow-derived mesenchymal stem cell
Ca^{2+}	calcium
cDNA	complementary DNA
ChAT	choline acetyltransferase
CNGF	ciliary neurotrophic growth factor
CNS	central nervous system
CO_2	carbon dioxide
CT	cycle threshold
CYC1	cytochrome c-1
DAPI	4',6-diamidino-2-phenylindole dilactate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EMEM	Eagle's minimum essential medium
ERK	extracellular signal-regulated kinase
ET-1	endothein-1
FGF	fibroblast growth factor
GABA	γ-aminobutyric acid

GCL	ganglion cell layer
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLAST	glutamate–aspartate transporter
GLT-1	glutamate transporter
GLUL	glutamate-ammonia ligase
GS	glutamine synthase
HamF-12	Ham's nutrients mixture
HAT	histone acetyl transferase
HDAC	histone deacetylase
HO-1	heme oxygenase 1
HORC	human organotypic retinal culture
HSP	heat shock protein
IGF-1	insulin-like growth factor
IL	interleukin
INL	inner nuclear layer
iNOS	inducible nitric-oxide synthase
IOP	intraocular pressure
IPL	inner plexiform layer
JNK	Jun N-terminal protein kinase
kDa	kilodalton
LC	lamina cribrosa
LDH	lactate dehydrogenase
LIF	leukaemia inhibitory factor
MAPK	mitogen-activated protein kinase
mmHg	millimetres mercury

MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
Na^+	sodium
NAD^+	nicotinamide adenine dinucleotide
NeuN	neuronal nuclei
NF	neurofilament
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFL	nerve fibre layer
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxyde
NOS	nitric oxide synthase
NRR	neuroretinal rim
NSC	neural stem cell
NTG	normal tension glaucoma
O_2	oxygen
OCT	optimal cutting temperature
OGD	oxygen glucose deprivation
OH	ocular hypertension
ON	optic nerve
ONH	optic nerve head
ONL	outer nuclear layer
OPL	outer plexiform layear
PACG	primary angle closure glaucoma

PARP	poly ADP ribose polymerase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PGE	prostaglandins
PI3K	phosphatidylinositol 3-kinase
POAG	primary open angle glaucoma
PSS	physiological saline solution
QRT-PCR	quantitative real-time polymerase chain reaction
RARB	retinoic acid receptor β
RGC	retinal ganglion cell
RNA	ribonucleic acid
RNFL	retinal nerve fibre layer
ROS	reactive oxygen species
RPE	retinal pigment epithelium
RTK	receptor tyrosine kinase
S.E.M	standard error of the mean
SF	serum free
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
TESPA	3-aminopropyl-triethoxyl saline
TGF	transforming growth factor beta
TM	trabecular meshwork
TNF	tumour necrosis factor
TOP1	topoisomerase 1
TRAIL	TNF-related apoptosis-inducing ligand
TrKB	tropomyosin-related kinase B

TSA	trichostatin A
TUNEL	terminal deoxynucleotidyl transferase-mediated biotindeoxyuridine triphosphate nick end-labelling
VEGF	vascular endothelial growth factor
VF	visual field
VPA	sodium valproate
UK	United Kingdom
USA	United States of America

Acknowledgments

I am most grateful to my supervisors, Dr Julie Sanderson and Professor David Broadway, for giving me an opportunity to develop myself as a rounded clinician for the benefit of my patients. Undertaking my own research has been a challenge, but it was also a pleasure to discover qualities in myself that have allowed me to become more focused and revealed new perspectives for my future. Julie Sanderson was always there to guide me through the labyrinth of scientific research. David Broadway was an invaluable source of encouragement enabling me to complete the work.

I would like to express my gratitude and love for my family, especially my son Alexander who never complained and was always there to give me a hug. Without their support I would never have finished this work. I hope that my achievement would encourage him to be never afraid of a good challenge. My husband, Julian, was always there for me and took care of our family when I was not with them. I dedicate this thesis to them.

I also would like to thank my colleagues from the eye department who always supported me in my research, particularly Mr Narman Puvanachandra, Mr Tom Eke, Mr Colin Jones and Mr Nuwan Niyadurupola for their invaluable advice, as well as Dr Varajini Joganathan and Dr Deepak Gupta for their friendship.

I am also very grateful to the people who supported me while I learnt the research techniques in the eye lab. I would like to express a special thanks to Dr Michael Wormstone, who was always there to answer a question and have a nice chat, Dr Andrew Osborne, who was a Ph.D student at the time and I learnt a lot from him, Dr Julie Eldred and Dr Sarah Russell were always happy to take time to explain if I was unsure. I am very grateful to other people from the Norwich research lab who made my time special Dr Jeremy Rhodes, Amal Al-Darwesh, Phillip Wright, Dr Andrew Smith, Richard Evans-Gowing, Sarah Gardner, Simon Ball, Dr Ning Ma, Dr Hanruo Liu, and Dr Chunlai Fang.

It is also important to mention the hard work by people from the Norwich Eye Bank, Pamela Keeley and Mary Tottman, because without them any of the work performed in our lab would not be possible.

I am deeply grateful to my parents for always supporting me in my determination to develop myself as a better person in life and at work. I think my mum spent a fortune on candles in our local church during my research.

CHAPTER 1

INTRODUCTION

The eye

Anatomy and physiology

The eye is a highly specialised photosensory organ. The main function of the eye is to process visual signals. Light is focused on the retina, where it is converted into electrical signals and then sent to the brain by means of the retinal ganglion cell axons. The eye has an approximate shape of a sphere with an average axial length of approximately 24mm and a volume of about 6.5ml (Forrester *et al.* 2008). However, short sighted eyes have a longer axial length, whereas long-sighted eyes are shorter than average.

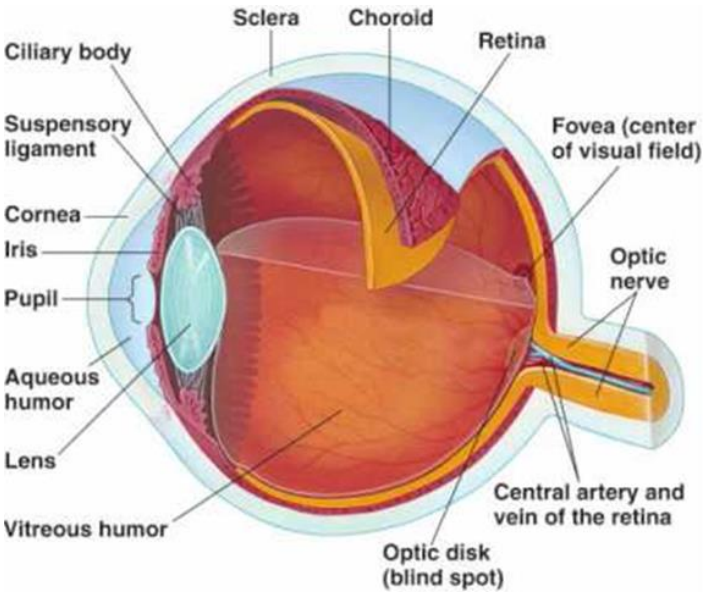


Figure 1: The eye anatomy. Adapted from https://www.eyesite.co.uk.

The globe of the eye consists of three main layers. The outer layer comprises the cornea and sclera. The middle vascular layer consists of the choroid, ciliary body and the iris. The innermost neurosensory layer is the retina. The cornea is the transparent anterior part of the eye which allows light to enter the eye to be focused on the retina. The cornea consists of several layers: the corneal epithelium, the stroma, Descemet's membrane and the endothelium. Together with the pre-corneal tear film, the cornea is responsible for most of the refractive power of the eye. The sclera is the non-transparent, white part of the outer coat of the eye. The main roles of the sclera are supportive to and protective of the ocular contents. The transparency of the outer layer of the eye depends on the thickness and the arrangement of the collagen fibres, together with the water content. In the cornea, the collagen fibres are highly organised and the water content is very low. In contrast, in the sclera collagen fibres are chaotically arranged, have different diameters and the water content is high. The thickness of the sclera varies considerably in different parts of the eye, being thickest (approximately 1mm) at the most posterior aspect of the eye (Forrester *et al.* 2008). The region of the sclera where the optic nerve exits the eye is known as the *lamina cribrosa* (LC). The LC is a round sheet of collagen fibres with multiple perforations through which axons of the retinal ganglion cells (RGCs) exit the eye to form the optic nerve (ON). The LC provides support for the RGC axons, but is weaker than the much thicker and denser surrounding sclera (Snell & Lemp 1989).

Light entering the eye is focused on the retina by the lens. While the lens has less refractive power than the cornea, in the younger individual it is able to

change its shape and thus the direction of the refracted light and is responsible for the fine focusing of light. The lens is made up of long hexagonal cells and surrounded by an elastic capsule. The lens is transparent in young individuals, but with age this quality is gradually lost, ultimately leading to cataract development. The lens is suspended in position by delicate fibres, called zonules, which connect the lens to the ciliary body.

The space in front of the lens is divided into anterior and posterior chambers by a pigmented, contractile structure called the iris. The iris acts as a diaphragm which controls the exposure of the retina to light through its opening, the pupil. The iris has a vascular structure supported by collagen stroma and pigmented cells, melanocytes. The size of the pupil is regulated by counteraction of the dilator and the sphincter pupillae muscles.

The ciliary body is a part of the vascular, middle coat of the eye and is positioned circumferentially at the base of the iris. The shape of the lens is changed by the movement of the muscles within the ciliary body, increasing refractive power is being referred to as accommodation.

The space behind the lens, known as the vitreous cavity, is filled with vitreous humour that acts like a cushion supporting the lens and protecting the retina. The vitreous has the consistency of a viscoelastic gel. The vitreous gel is transparent and is composed mainly of water (98%), fine collagen fibres and hyaluronic acid (Forrester *et al.* 2008).

The choroid is a highly vascular and heavily pigmented layer, positioned between the retina and the sclera. The main function of the choroid is to supply

blood to the retina. Because of heavy pigmentation, the choroid also helps to minimise visual disturbances by absorbing light and preventing it reflecting back into the eye.

Retinal structure and function

Topographic organisation

The retina is divided into central and peripheral regions. The central region of the retina, called the macula, is surrounded by the superior and inferior temporal branches of the central retinal artery and vein. The average diameter of the macula is about 5.5mm (Snell & Lemp 1989).

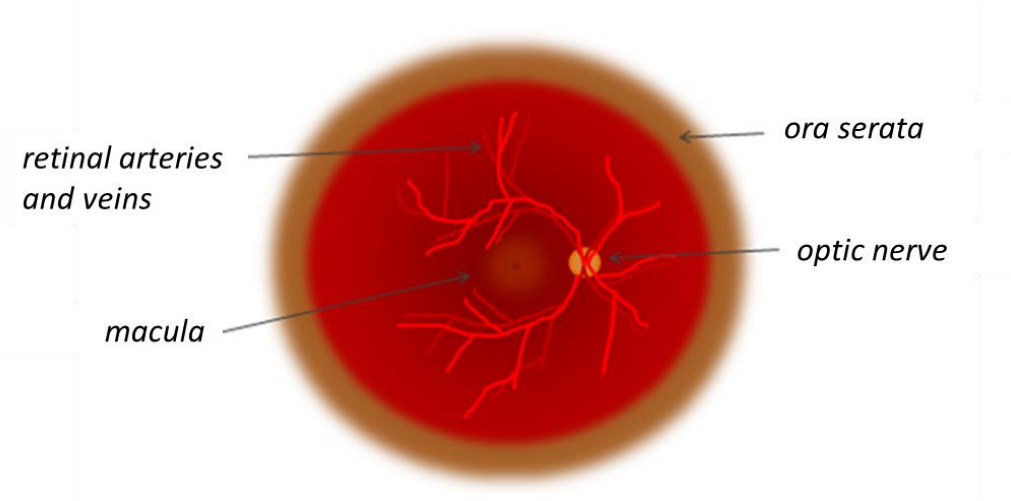


Figure 2: Schematic diagram of the retinal topography.

The most central point of the macula, called the *foveola*, has no retinal blood vessels or RGCs and is responsible for the highest resolution visual acuity. The area around the *foveola*, about 0.35mm in diameter, has the thickest layer of RGCs and is called the *fovea*. The presence of yellow carotenoid pigments, zeaxanthin and lutein, in the cone photoreceptors gives the region its name –

the *macula lutea (*Bernstein *et al.* 2001*).* Macular pigment absorbs short-wavelength (blue) light (Snodderly *et al.* 1984), thereby affording protection from acute light damage (Barker *et al.* 2011). Moreover, macular pigment offers protection against oxidative stress to the retina (Khachik *et al.* 1997).

In relation to the central retina, the head of the optic nerve is located slightly toward the nasal area, about 3mm from the centre of the macula. The average diameter of the optic nerve head is about 1.5mm (Snell & Lemp 1989). The central retinal vessels arise from the optic disc and divide into superior and inferior branches followed by further division into nasal and temporal branches according to the retinal anatomy. The peripheral retina comprises the remaining retina outside the temporal retinal vessels. The most peripheral area of the retina, called the *ora serata*, is a transition zone where the retina meets the ciliary body.

The cellular structure and function

The retina is the neurosensory, inner layer of the eye where light is converted into electrical signals. The retina has a complex structure with highly organised layers of cell bodies and inter-cellular connections. Retinal thickness varies throughout the tissue. Close to the optic nerve head, the retinal depth is approximately 0.56mm, whilst at the *ora serrata* it is only 0.1mm (Snell & Lemp 1989). All RGC axons project towards the LC to form the optic nerve head, such that the retinal nerve fibre layer (RNFL) thickness is most prominent in that area. Light travels though the retina before being absorbed by photosensitive cells, the cones and rods. The electrical response generated by photoreceptors is transmitted and modified by means of interneurons (bipolar,

amacrine and horizontal cells) to the RGC bodies and then to the brain via RGC axons. Glial cells support the retinal tissue structure and maintain the retinal microenvironment.

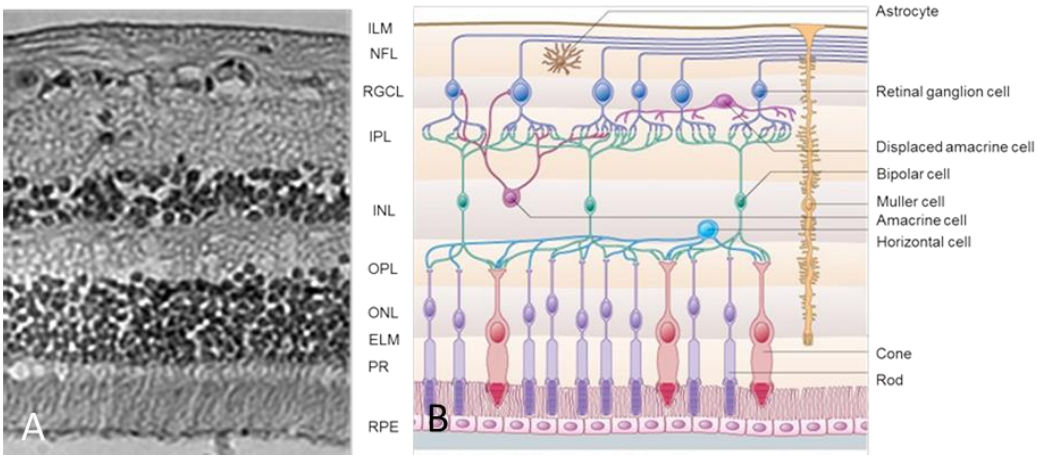


Figure 3: The layers and cellular organization of the retina. (A) Low-power micrograph (H&E staining); (B) Schematic diagram. ILM=internal (inner) limiting membrane, NFL=nerve fibre layer, RGCL-retinal ganglion cell layer, IPL=inner plexiform layer, INL=inner nuclear layer, OPL=outer plexiform layer, ONL=outer nuclear layer, ELM=external (outer) limiting membrane, PR=photoreceptors outer segments, RPE=retinal pigment epithelium. Image B is adapted from http://webvision.med.utah.edu/imageswv/schem.jpeg & husect.jpeg.

Retinal Ganglion Cells

RGCs are neurons that transmit visual signals from the retina to the brain. RGC structure consists of a cell body, multiple short dendrites and one long axon. The RGC bodies are located in the innermost nucleated layer called the RGC layer of the retina. The RGC dendrites connect with the bipolar and amacrine cells from the inner nuclear layer (INL) forming the inner plexiform layer (IPL). There are about 1.2 million RGCs in the retina of each human eye (Forrester *et al.* 2008). The thickness and density of the RGC layer is not

uniform throughout the retina. There are up to seven rows of RGC nuclei in the perifoveal region, with an average thickness from 60 to 80µm (Forrester *et al.* 2008). Outside the macula, the RGC layer decreases to a single row, with an average thickness from 10 to 20 µm (Forrester *et al.* 2008). RGCs are absent from the *foveola*. The macula contains around 60% of all RGCs in the retina, even though it only occupies less than 1.4% of retinal surface (Curcio & Allen 1990).

RGC axons travel toward the optic nerve head within the RNFL, passing through the LC to subsequently become myelinated by oligodendrocytes. On the way to the visual cortex of the brain, RGC axons form the optic nerve and synapse with cells in the lateral geniculate nuclei via the optic chiasm and the optic tract. The axons of the cells from the lateral geniculate nuclei form optic radiations within the parietal lobes of the brain. Finally, electrical signals from the retina are delivered to the primary visual cortex in the occipital lobes, where they are analysed and expressed as visual perception.

Retinal ganglion cell classification

RGCs are classified according to cell morphology and function. Conventionally, two classes of RGC have been identified in primates, the midget and parasol RGCs, classified according to size of the dendritic tree, receptive field diameter and speed of the signal conduction. RGCs of the midget system are quite small, whereas the parasol cells are much larger, with their dendritic tree and receptive field diameters being approximately three times greater than that of the midget RGCs. Axons of the parasol RGCs are larger and have faster conduction velocities compared with that of the midget

RGCs (Watanabe & Rodieck 1989). However, because of the development of new research techniques, it is believed now that there are at least 20 types of RGCs (Masland 2012). Based on spatial distribution of the mouse RGC dendritic trees, Sumbul and colleagues (2014) have classified RGCs into 15 types by combining evidence from light and electron microscopy together with genetic labelling (Sumbul *et al.* 2014). A recently discovered RGC type is the intrinsically photosensitive retinal ganglion cell (ipRGC). IpRGs contain the photopigment melanopsin and are responsible, in part, for the pupil response to light and circadian cycles (Sand *et al.* 2012). IpRGCs comprise only 0.2% of total retinal ganglion cells in the eye (Dacey *et al.* 2005).

Functionally, RGCs are classified based on the response to light as ON, OFF and ON/OFF RGCs. ON-ganglion cells fire in response to light, whereas OFF-ganglion cells have an opposite response to light stimulation. ON/OFF RGCs respond to both the onset and the termination of light (Kuffler 1953). Each ganglion cell is sensitive to illumination of a certain size, the area being called the receptive field of the RGC. The receptive field of each RGC is composed of two concentrically arranged regions: an excitatory centre and an opposite surrounding region. The opponent centre/surround organisation of the RGCs receptive field improves vision quality, specifically colour contrast and focus enhancement (Forrester *et al.* 2008).

Interneurons (bipolar, amacrine and horizontal)

The fundamental functions of interneuron cells are to transmit and modify the electric signal sent from photoreceptors towards the RGCs, therefore enhancing contrast sensitivity and image quality. Horizontal cells modify synaptic

transmission between photoreceptors and bipolar cells whereas amacrine cells modify transmission between bipolar and RGCs (Masland 2001). For example, horizontal cells can slow down the transmission of signals by releasing inhibitory neurotransmitters, mainly γ-aminobutyric acid (GABA). Feedback from horizontal cells to photoreceptors enables adjacent regions of the retina to compare the intensity of light, therefore enhancing the perception of contrast (Masland 2001). Amacrine cells, on the other hand, can be GABAergic or can release excitatory neurotransmitters, for example dopamine orü acetylcholine**.** Bipolar cells directly connect photoreceptors to RGCs. In the macula, cone bipolar cells may make contact with as few as one cone in the central retina, whilst peripheral retinal rod bipolar cells may receive inputs from up to 70 rods. There are two types of bipolar cells, called ON and OFF, depending on their response to glutamate. In the dark, glutamate is released by photoreceptors and stimulates hyperpolarization of ON and depolarization of OFF bipolar cells. Light reduces glutamate availability, thereby controlling the polarized state of cells. The quality of the image is enhanced by the difference in response to light by both types of bipolar cells (Masland 2001). The interneuron cell bodies are grouped within the INL, and their dendrites form synaptic connections with RGCs and photoreceptors in the inner and outer plexiform layers.

The retinal glia (Müller cells, astrocytes and microglia)

Müller cells are the main supporting cells of the retina. The long cell bodies of Müller cells extend from the inner edge of the retina, where their foot processes lie adjacent to the inner limiting membrane, running through the entire depth of the retina to form the outer limiting membrane with the inner segments of the

photoreceptors. In addition to their mechanical function, Müller cells produce and secrete various trophic factors, including leukaemia inhibitory factor (LIF) (Joly *et al.* 2008, Von Toerne *et al.* 2014), osteopontin (Del Rio *et al.* 2011) and transferrin (Picard *et al.* 2008).

Astrocytes are predominant in the RNFL, RGC layer and at the optic nerve head, forming an irregular scaffold between neurons, neuron synapses and vessels (Chu *et al.* 2001, Trivino *et al.* 1997). Besides maintaining structural integrity of the retina, astrocytes are also involved in various important functions including promotion of glutamate clearance, potassium buffering, antioxidant defence and homeostasis of purines (ATP and adenosine), GABA and D-serine (Allen & Barres 2009, Franke & Illes 2014, Larsson *et al.* 1980, Levi & Patrizio 1992). Astrocytes produce and release various molecular mediators, including prostaglandins (PGE), nitric oxide (NO) and arachidonic acid (AA), that are involved in the regulation of blood supply to the retina through the control of blood vessel diameter (Iadecola & Nedergaard 2007).

Retinal microglial cells are similar to brain macrophage-like phagocytic microglial cells and their main roles relate to tissue homeostasis and immune responses (Forrester *et al.* 2008).

Photoreceptors

Photoreceptors are responsible for the absorption of light by means of visual pigment and initiation of neuro-electrical impulses. Photoreceptor cells are divided into two morphologically distinctive groups: rods and cones. Rods are responsible for contrast, brightness and motion, whereas cones specialize in fine and spatial resolution together with colour vision. In the human eye, there

are about 115 million rods and only 6.5 million cones (Forrester *et al.* 2008). With respect to photoreceptors, the macula is populated almost entirely by cones, whereas the peripheral retina is populated mainly with rods. Rods contain the visual pigment rhodopsin, which absorbs blue-green light (Brown & Wald 1964). Rod photoreceptors are highly sensitive and are active in dark-dim light conditions. Cones contain opsins as their visual pigments. Depending on the structure of the opsin molecule, specific cones are sensitive to long (red), medium (green) or short (blue) wavelengths of light. The presence of cones of different wavelength sensitivity provides the retina with the basis for colour perception. In dim light, perceived images appear rather grey in colour since rods are "colour blind" photoreceptors.

The retinal pigment epithelium

The retinal pigment epithelium (RPE) is a pigmented monolayer of hexagonal shaped cells positioned between the photoreceptors and the underlying choroid. The RPE closely interacts with the photoreceptors for the maintenance of visual function. RPE cells are densely packed with granules of melanin that absorb lights and reduce light scatter within the eye. A major function of the RPE is the regeneration of 11-cis-retinal through a multistep enzymatic pathway known as the retinoid visual cycle (Driessen *et al.* 2000). The RPE is also involved in the disposal of the outer segment of photoreceptor cells (Finnemann 2003). The RPE takes up nutrients such as glucose, retinol, and fatty acids from the choroidal blood and delivers these to the photoreceptors, as well as removing ions, water, and metabolic end products from the subretinal space into the choroidal blood for their clearance (Strauss 2005). In addition,

the RPE secretes a variety of growth factors, including vascular endothelial growth factor (VEGF) (Adamis *et al.* 1993) and platelet-derived growth factors (PDGFs) (Campochiaro *et al.* 1994). The RPE also secretes intercellular matrix helping to maintain the structural integrity of the retina and the choroid (Forrester *et al.* 2008).

The optic nerve head

The optic nerve head (ONH) is an area of the retina where all RGC axons leave the globe to form the optic nerve (ON). The edge of the ONH is raised, because of a large number of the RGC axons that are grouped together, and is called the neuroretinal rim (NRR), while the central area is depressed. Within the central depression of the ONH, the central retinal vessels enter and leave the eye and the region of the depression is called the ONH cup. Axons of the macular RGCs form about 90% of the NRR (Forrester *et al.* 2008). The RGC axons exit the eye via perforations in a round sheet of collagen fibres known as the *lamina cribrosa* (Snell & Lemp 1989). Astrocytes are the major glial cell type in the nonmyelinated ONH in most mammalian species and are oriented perpendicular to the RGC axons (Hernandez 2000). In the ONH, astrocytes not only provide structural and functional support to the axons, but they also form the interface between connective tissue surfaces and surround blood vessels (Anderson 1969). Due to absence of both the retina and the choroid in the region of the ONH, the ON area is functionally represented as 'the blind spot' in the visual field of the eye. As ganglion cell axons pass through the LC, they become myelinated by oligodendrocytes, resulting in a doubling of the thickness of the ON (Forrester *et al.* 2008).

Blood supply to the retina and the optic nerve head

The retina is one of the most metabolically active tissues in the human body and it has been estimated that it consumes more oxygen per gram of tissue than the brain (Ames 1992). Although the blood supply of the retina is provided by several sources, there are anatomical pitfalls that make the retina extremely vulnerable to circulatory disturbances. The supply of nutrients and clearance of end products is provided by two major sources: the central retinal and the choroid vessels, both originating from the ophthalmic artery. In the retina, branches of the central retinal vessels nourish the inner two-thirds, while the choroidal circulation supplies the outer one-third. There is no connection between retinal and choroidal circulations within the retina, except at the optic nerve head, where a connection exists as a network of capillaries, called the circle of Zinn-Haller (Forrester *et al.* 2008). The central retinal artery divides into four major branches that supply corresponding retinal sectors. There is no overlap in the blood supply of these sectors, such that the inner retina has no alternative in maintaining its viability and thereby is limited in its ability to recover if retinal artery occlusion occurs.

Aqueous humour function and dynamics

Aqueous humour is the fluid that maintains the intraocular pressure (IOP) and the shape of the anterior segment of the eye. The aqueous supplies nutrients to the avascular structures of the eye, such as the lens and the cornea. Aqueous humour is composed of water (98%) and electrolytes, and its chemical composition is similar to plasma, except for a relatively low protein concentration. Significant aqueous humour components include glucose,

lactate, ascorbate, albumin, transferrin, fibronectin, immunoglobulins and enzymes (eg. carbonic anhydrase, lysozyme, plasminogen activator), cytokines (eg. basic fibroblast growth factor and transforming growth factor-β), catecholamines (adrenalin, noradrenalin and dopamine), glutathione and hyaluronic acid (Forrester *et al.* 2008). Aqueous humour is produced continuously by the non-pigmented epithelium of ciliary body processes and passes through the pupil (from the posterior chamber into the anterior chamber) towards the circumferential irido-corneal drainage angle, from where it leaves the eye through two main outflow routes (Lewis *et al.* 1999). The conventional outflow route is via the trabecular meshwork (TM) into Schlemm's canal. Aqueous humour reaches venous systems by means of collector channels and aqueous veins. The drainage angle is an anatomical structure positioned between the cornea and the iris. The non-conventional or uveoscleral outflow route is when drainage occurs through the iris root into the space between the sclera and the choroid. Elevated IOP can be caused by an increased resistance of aqueous outflow through the TM (Johnson 2006).

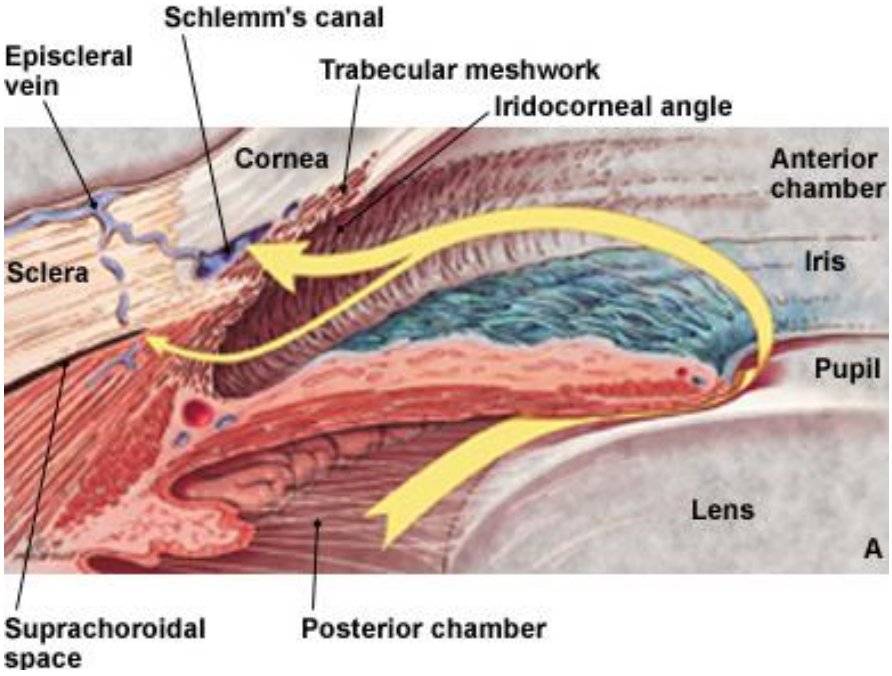


Figure 4: Aqueous humour outflow. Normal outflow through trabecular meshwork (large arrow) and uveoscleral routes (small arrow) and related anatomy. Most aqueous flow is through the trabecular meshwork. Each pathway is drained by the eye's venous circulation. The image is adapted from Lewis et al. 1999.

Glaucoma

Background, diagnosis and classification

Glaucoma is the second leading cause of blindness worldwide with an estimated 60.5 million people affected (Quigley & Broman 2006). It is a neurodegenerative disease, where the gradual loss of RGCs causes progressive visual field (VF) loss, which can result in tunnel vision and eventual blindness. Clinically, the diagnosis of glaucoma is based on progressive thinning of the optic nerve head NRR and corresponding characteristic VF defects. According to the IOP level, glaucoma is classified into two major sub-types: high tension, primary open angle glaucoma (POAG) and normal tension glaucoma (NTG).

The normal population IOP is considered to be between 10 and 21mmHg. The normal range has been established statistically, and is based on two standard deviations above/below the mean IOP of 16 mmHg in normal population (Hollows & Graham 1966). There are other types of glaucoma based on the cause of high IOP. Glaucoma can be classified as primary or secondary, congenital or acquired. Angle closure glaucoma is caused by a physical obstruction of the drainage angle for aqueous outflow. Secondary glaucoma occurs as a consequence of a different primary condition such as uveitis, trauma or neovascularisation. Elevation of IOP with no associated structural change to the NRR of the optic nerve head, or functional deterioration on VF testing is called ocular hypertension (OH).

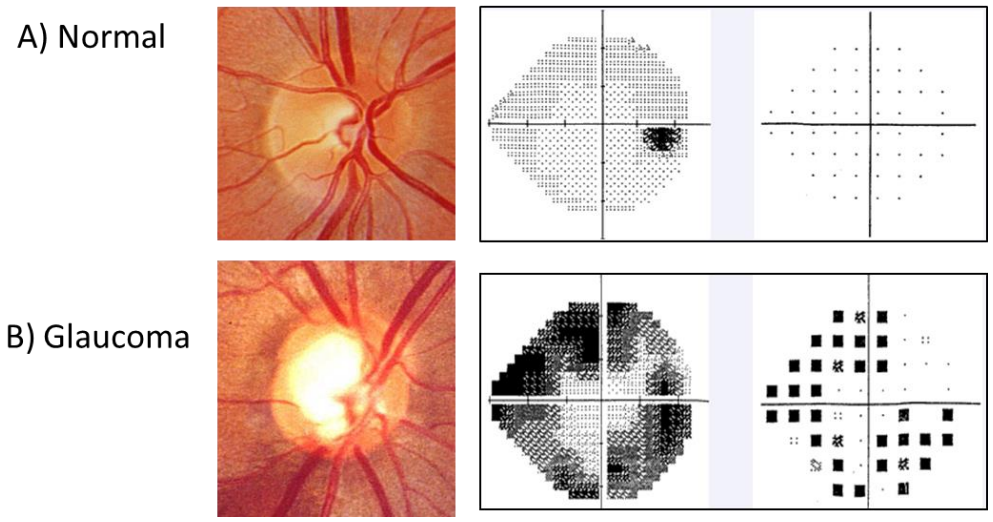


Figure 5: An example of structural and functional changes in a patient with NTG. A) Healthy appearance of the optic nerve head neuroretinal rim with normal visual field. B) Glaucomatous neuroretinal rim loss and tunnel-like visual field defect.

Risk factors

Glaucoma is a complex disease caused by a combination of risk factors, the main one of which is IOP. IOP is the main treatable risk factor and several population-based studies have shown an association of glaucoma prevalence and high initial IOP (Dielemans *et al.* 1994, Sommer *et al.* 1991). A significant reduction of IOP (>20%) halves the risk of glaucoma development in patients with OH (Kass *et al.* 2002). Although, in the majority of cases, glaucoma progression cannot be halted, it has been shown that a reduction of IOP slows down the progression of the disease in eyes with either POAG or NTG (Advanced Glaucoma Intervention Study (AGIS)2000, Collaborative Normal Tension Glaucoma Study 1998). However, some patients gradually deteriorate despite a significant drop in IOP on treatment (Heijl *et al.* 2002). Age is another major contributing factor in glaucoma development. Multiple population-based studies have shown a rapid rise in the prevalence of glaucoma in individuals over 50 years of age (Dielemans *et al.* 1994, Heijl *et al.* 2013, Leske *et al.* 1994). Family history of glaucoma is another important factor, since both first- and second-degree relatives are at a significantly higher risk for glaucoma development, the relative risk being more than ten-times greater among first-degree relatives of affected patients with POAG (Wang *et al.* 2010b, Wolfs *et al.* 1998). Recent advances in understanding of the human genetic code have identified several genes, such as myocylin (*MYOC*), optineurin (*OPTN*), cytochrome *P4501B1* (*CYP1B1*), apolipoprotein E gene (*APEO*), and latent transforming growth factor-β binding protein 2 (*LTBP2*) which provide foundation for future research in glaucoma genetics (Ali *et al.*

2009, Copin *et al.* 2002, Fingert *et al.* 1999, Hollander *et al.* 2006, Rezaie *et al.* 2002, Stoilov *et al.* 1997). Race is also a significant risk factor, with people of Asian and African-descent being at a significantly greater risk of developing glaucoma compared with Caucasians (Stein *et al.* 2011, Tielsch *et al.* 1991). The incidence of primary angle closed glaucoma (PACG) is known to be higher in patients of Chinese and Eskimo origin compared to that in European and African populations (Congdon *et al.* 1992, Congdon *et al.* 1997, Foster & Johnson 2001). It has been shown that individuals with myopia are more likely to develop open angle glaucoma, with a direct correlation of the risk with an increase in the refractive error (Marcus *et al.* 2011). Hypermentropia, on the other hand, is a common finding in patients with PACG (Lowe 1970). A history of vasospasm, such as migraine or cold extremities (Raynaud's phenomenon) and a nocturnal reduction in systemic blood pressure have been identified as contributing factors for glaucoma progression in patients with NTG and POAG (Broadway & Drance 1998, Drance *et al.* 2001, Graham & Drance 1999, Leske 2009). Identification of high risk patients is important with respect to screening. Close monitoring for detectable ON damage and/or progressive VF loss helps clinicians recommend treatment as early as possible in order to slow down the pathological changes.

Pathophysiology

There are big gaps in our understanding of the pathological processes involved in glaucoma development and progression. It is most likely that a combination of mechanical (Burgoyne *et al.* 2005) and metabolic factors (Drance *et al.* 2001) triggers an initial assault on RGCs and their surroundings. It is possible that

with time some processes initially intended to be protective, become detrimental to RGC survival causing further damage to partly diseased RGCs, their neighbouring cells in the retina (secondary neuro-degeneration) and the visual pathway within the brain (Levkovitch-Verbin *et al.* 2003). Some poorly understood factors, for example age-related changes in structure and function of vascular (Grunwald *et al.* 1993) and connective tissue or genetic abnormalities, may act as predisposing elements for the damage in glaucoma, making some patients uniquely susceptible to glaucoma development (Advanced Glaucoma Intervention Study (AGIS) 2000).

Intraocular pressure

It is well established that the level of IOP is the major risk factor in the aetiology of glaucoma. In man and other primates, the mechanical forces induced by elevation of pressure on the ONH, as the weakest point within the tough scleral layer of the globe, cause a displacement of the mesh-like connective tissue of the LC outwards (figure 6) (Burgoyne *et al.* 2005, Quigley 1999, Quigley & Addicks 1981). The mechanical compression of the unmyelinated RGC axons passing through the narrow spaces within the collagen plates of the LC leads to disruption of the axoplasmic flow and subsequent RGC axon degeneration, both retrograde and anterograde (Anderson & Hendrickson 1974, Minckler *et al.* 1977). Evidence for disrupted axoplasmic flow in glaucoma has been shown in rats where the retrograde flow of radioactively labelled brain-derived neurotrophic factor (BDNF) from the synapse of the retinal ganglion cells to the cell body has been shown to be inhibited by an acute elevation of IOP (Quigley *et al.* 2000). Clinically, the

pathological processes in humans lead to a characteristic glaucomatous "cupping" of the ONH associated with a specific pattern of VF loss (figure 6).

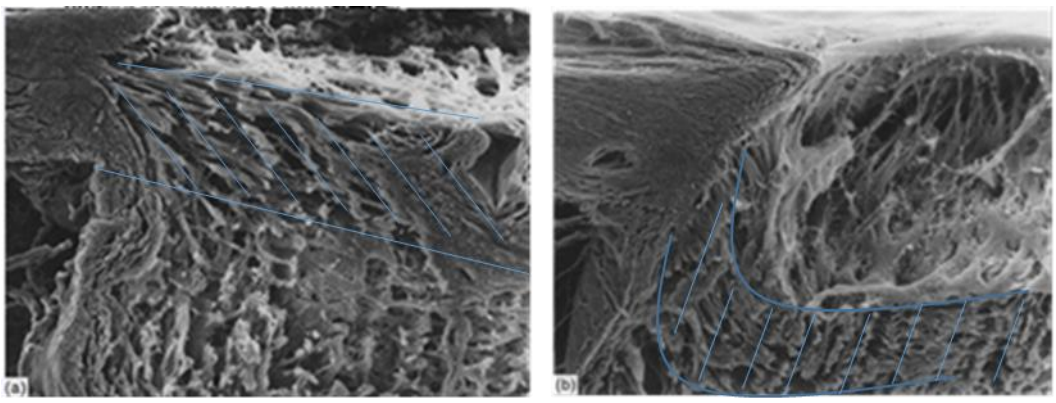


Figure 6: Scanning electron micrographs illustrate the normal position of the *lamina cribrosa* (blue lines) in a human eye (a) and the characteristic of glaucoma excavation of the ONH (b). Adapted from Quigley 1999.

Similar changes to those observed in humans are seen in smaller mammalian species, such as rat and mouse, where the predominant cellular elements of the ONH are the astrocytes rather than collagen fibres (Dai *et al.* 2012). Dai and colleagues have proposed that the ONH astrocytes are the primary targets of the mechanical forces induced by the glaucomatous condition (Dai *et al.* 2012). Together with the loss of the RGC support, the authors have suggested that the effect on RGCs is metabolic. For example, the energy metabolism of the RGC is dependent on glycolytic products, in the form of lactate, released by astrocytes (Tsacopoulos & Magistretti 1996, Wender *et al.* 2000) because neurons use glucose to maintain their antioxidant status at the expense of its utilization for bioenergetic purposes (Herrero-Mendez *et al.* 2009). By

removing potassium and glutamate (Choi *et al.* 2012, Farinelli & Nicklas 1992, Magistretti *et al.* 1999, Waniewski & Martin 1986) from the extracellular space, astrocytes maintain the extracellular environment for neighbouring neurons (Rosenberg 1991). By providing various neurotrophic factors, such as nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-α and TGF-β, BDNF and glial cell line-derived neurotrophic factor (GDNF), astrocytes exert a neuroprotective function (Buchanan *et al.* 2000, Duenas *et al.* 1994, Flores *et al.* 1999, Fonseca *et al.* 2014, Lu *et al.* 2014, Wu *et al.* 1998). Loss of astrocyte function has the potential, therefore, to result in multiple adverse effects on RGC function.

Glutamate cycle and excitotoxicity

Glutamate is the most abundant free amino acid found in the brain (Schousboe 1981) and is considered to be the major neurotransmitter in the mammalian central nervous system. Glutamate is involved in most aspects of normal brain function including cognition, memory and learning, as well as regulation of brain development and cell survival, differentiation and function of synapses (Danbolt 2001). Glutamate exerts its signalling function by binding to and thereby activating glutamate receptor proteins. Several subtypes of glutamate receptor have been identified: the ionotropic receptors, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors and metabotropic receptors (mGluR). Activation via the ionotropic NMDA receptor is believed to be the most efficient at causing cell death (Arundine & Tymianski 2003). There are no specific

enzymes for glutamate break-down present in the extracellular space, so that control of glutamate receptor activation is related to the balance between release of glutamate into the extracellular fluid and the removal of glutamate from it. Extracellular glutamate overload leads to over-activation of the glutamate receptors which in turn result in prolonged and massive depolarization, excessive influx of Ca^{2+} into neurones (Sanchez-Armass & Blaustein 1987), as well as Ca^{2+} release from the endoplasmic reticulum (Pin & Duvoisin 1995). As a results of high intracellular Ca^{2+} concentration, calcium-dependent processes are also inappropriately activated, including proteases, caspases, lipases, endonucleases, pro-apoptotic factors and the production of free radicals from the mitochondria, consequently causing cell death (Arundine & Tymianski 2003, Lipton & Nicotera 1998). In order to maintain physiological glutamate concentrations, rapid glutamate uptake is catalyzed by a family of Na^{+}-dependent transporter proteins, such as glutamate–aspartate transporter (GLAST) (EAAT1 in humans), glutamate transporter (GLT-1) (EAAT2 in humans), excitatory amino acid carrier 1 (EAAC1) (EAAT3 in humans) and excitatory amino acid transporter 4 and 5 (EAAT4 and EAAT5 in humans), located at the cell surface of both astrocytes and neurons (Danbolt 2001, Grewer & Rauen 2005, Marcaggi & Attwell 2004). In 1994, Pellerin and Magistretti proposed that the glutamate released from neurons as a result of increased synaptic activity is taken up by astrocytes where it is converted to glutamine by glutamine synthase (GS). Glutamine is then transported out of the astrocyte into the neuronal pre-synaptic terminal via the extracellular space, where it is converted by glutaminase to glutamate (Pellerin & Magistretti 1994).

In the retina of rats and mice, antibodies to GS were found to be localized to the Müller cells and astrocytes (Anlauf & Derouiche 2013, Chang *et al.* 2007, Derouiche & Rauen 1995, Riepe & Norenburg 1977).

The first evidence of glutamate toxicity to retinal neurons came from the experiment on newborn mice, where the subcutaneous injection of monosodium glutamate destroyed the neurons in the inner layers of the retina (Lucas & Newhouse 1957). Since then, other studies have confirmed that high doses of, or prolonged exposure to, glutamate causes RGC death via stimulation of ionotropic glutamate receptors (Bai *et al.* 2013, Siliprandi *et al.* 1992, Sisk & Kuwabara 1985, Vorwerk *et al.* 1996). An excessive level of glutamate in glaucoma pathogenesis has been proposed and reported as a feature of glaucoma in monkeys, humans (Dreyer *et al.* 1996), and dogs (Brooks *et al.* 1997). However, Dalton in 2001 published a paper exposing Dreyer and his co-authors in data misconduct and subsequent investigations of allegations of missing data by the Harvard university, the Office of Research Integrity (ORI) and the Food and Drug Administration (FDA) (Dalton 2001). In rats, elevated glutamate levels in the vitreous has been documented in an IOP-induced retinal ischaemia model (Lagreze *et al.* 1998), whereas in an optic nerve trans-section model, there was no evidence of such an effect (Levkovitch-Verbin *et al.* 2002a). The concept of a toxic neuro-excitatory element to glaucomatous optic neuropathy is an attractive one that might, in part, also explain the processes involved in secondary neuro-degeneration of RGCs that are neighbours to others already affected by glaucoma (excitotoxicity) (Levkovitch-Verbin *et al.* 2003).

Dysfunction of glutamate transporters is often a key component of the cascade leading to excitotoxic cell death with subsequent release of intracellular glutamate (Attwell *et al.* 1993) and the potential for secondary excitotoxic neuro-degeneration. In the retina, glutamate uptake is mainly operated by the glial glutamate transporter GLAST and the neuronal transporter GLT-1 (Rauen 2000). The localisation of GLT-1 on neurons is only observed in retina after exposure to elevated IOP, where photoreceptors are consistent in their immunoreactive to GLT-1 antibodies (Sullivan *et al.* 2006). In the rat laser-induced model of ocular hypertension, some authors have found a significant reduction in the levels of both GLAST and GLT-1 (Martin *et al.* 2002), whereas others showed a sustained increase in GLAST expression (Woldemussie *et al.* 2004). Moreover, Sullivan and colleagues (2006) found no change in the level of GLAST, however the expression of GLT-1 was vastly altered in glaucomatous retinas from rats and humans (Sullivan *et al.* 2006). In a rat retinal ischaemia model, a decrease of neuronal glutamate uptake has been associated with a significant modulation of GLT-1 expression with no significant change in the level of GLAST (Russo *et al.* 2013). The importance of GLAST transporter in glaucomatous RGC degeneration was highlighted when GLAST-knockout mice were shown to develop spontaneous RGC death and glaucomatous optic nerve degeneration without IOP elevation (Harada *et al.* 2007).

Apoptosis

Apoptosis is a regulated cellular death process that involves multiple enzymes and exerts itself as a distinct sequence of morphological changes to affected

cells. Apoptosis is critical for normal tissue homeostasis and is involved in diverse processes including development and immune clearance. During apoptosis, cell content undergoes an internal degradation by means of condensation and shrinkage of the nucleus and cytoplasm. There is a distinction between two interlinked signalling pathways involved in apoptosis: the extrinsic, or receptor activated, and the intrinsic, or mitochondrial mediated pathways (Green 2000). Both pathways lead to cell death through the activation of proteases known as caspases. Initiator caspases are the first to be activated in the apoptotic pathway which, in turn, activate effector caspases, which trigger a series of proteolytic events that eventually lead to cell death (Fuentes-Prior & Salvesen 2004). The extrinsic pathway is based on stimulation of the death receptors (DRs) containing a special intracellular death domain on the cell surface. DRs mainly comprise the tumour necrosis factor (TNF) receptor family including Fas, TNFR1 and TRAIL (TNF-related apoptosis-inducing ligand) receptors, which become activated by their respective death ligands, such as FAS ligand, TNF and TRAIL (Ashkenazi & Dixit 1998). Stimulation of DRs follows activation of caspase-8, with subsequent proteolytic cleavage of caspase-3 which results in proteolytic cleavage of cytoskeletal and cell-cell adhesion proteins, chromatin condensation, and DNA fragmentation (Krammer *et al.* 2007, Wang *et al.* 2005), during which the cells form apoptotic bodies preventing leakage of cytoplasmic content. The apoptotic bodies attract phagocytic cells to clear the dead cell with minimal compromise to the surrounding tissue (Elmore 2007). The intrinsic pathway is more complex than the extrinsic pathway and is

known to be activated by an imbalance between levels of anti-apoptotic and pro-apoptotic B-cell lymphoma protein-2 (Bcl-2) family of proteins (Green & Kroemer 2004). The Bcl-2 protein family is divided into two classes of molecules that have opposing effects: antiapoptotic members such as Bcl-2 and Bcl-xL that protect the cell against apoptosis, and proapoptotic members such as Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) that trigger apoptosis (Lo *et al.* 2011). Pro-apoptotic proteins, Bax and Bak, together with a reactive oxygen species (ROS) (Chidlow *et al.* 2007) play a central role in changing mitochondrial membrane permeability, with subsequent release of cytochrome c (a component of the electron transport chain essential for cellular chemical energy). Cytochrome C binds to Apaf1 (pro-caspase apoptotic protease activating factor-1), forming an apotosome (Danial & Korsmeyer 2004), which in turn activates caspase 9 (Li *et al.* 1997), followed by activation of caspase-3 leading to initiation of apoptosis, DNA fragmentation and eventual cell death.

There is evidence that the loss of neurons in glaucoma occurs largely by apoptosis (Nickells 1999, Quigley 1999). It has been shown that both chronic elevated IOP and optic nerve axotomy can cause distinct morphological changes compatible with apoptosis in monkey and rabbit eyes when compared with controls (Quigley *et al.* 1995). Apoptosis of RGCs has been confirmed as a feature of glaucomatous neurodegeneration, for example, using *in vivo* rodent models of acute optic nerve injury (Berkelaar *et al.* 1994, Li *et al.* 1999), experimental ocular hypertension (Garcia-Valenzuela *et al.* 1995) and *in vitro* using the retina from patients with known glaucoma (Kerrigan *et al.* 1997,

Okisaka *et al.* 1997, Tatton *et al.* 2001), as wells as in non-glaucomatous conditions such as central retinal artery occlusion in rats (Zhang *et al.* 2005) and ischaemic optic neuropathy in humans (Levin & Louhab 1996).

Ischaemia

Ischaemia is a pathological condition caused by a shortage of blood supply, resulting in an inadequate delivery of oxygen and glucose. The eye as an organ is able to maintain a constant supply of blood in response to ocular perfusion pressure changes by controlling vascular resistance through changes in the diameter of the local vasculature, a process called autoregulation (Harris *et al.* 1998). There is a possibility that autoregulation is impaired in conditions caused by ischaemia, including glaucoma (Anderson 1999). It has been shown that not only patients with glaucoma have diminished blood flow in the NRR of the ONH when compared with OH patients and normal subjects, but the reduction in the NRR blood flow correlated with the size of the ONH cupping (Hafez *et al.* 2003). In general, patients with NTG have been found to have impaired ocular blood flow that appears to be more pronounced than in glaucoma patients with elevated IOP (Drance *et al.* 2001). Moreover, there is a vast amount of published data illustrating an association between systemic vascular pathology, such as atherosclerosis, arterial hypertension and hypotension, stroke, diabetes and glaucoma (Bonomi *et al.* 2000, Leske *et al.* 2002, Shoshani *et al.* 2012, Tielsch *et al.* 1995, Weinreb & Harris 2009). Specifically, studies have revealed a relationship between visual field defects and decreased blood flow velocities or increased resistance of the ophthalmic and short posterior ciliary arteries (Galassi *et al.* 2003, Martinez & Sanchez

2005). Reduction of the choroidal blood flow at the macula has been documented with increasing age (Grunwald *et al.* 1993), which in turn might eventually lead to age-related changes in the neuronal morphology (Balazsi *et al.* 1984) and could play a role in the aetiology of glaucoma. As a potential cause of reduced blood flow and relative ischaemia, high levels of the endothein-1 (ET-1), a powerful vasoconstrictor, have been found in the aqueous of glaucoma patients (Choritz *et al.* 2012, Tezel *et al.* 1997). Moreover, intravitreal injections of ET-1 have been shown to impair axonal transport in RGCs, as well as constricting the microvasculature of the optic nerve head and retina (Sasaoka *et al.* 2006, Stokely *et al.* 2002).

Oxidative stress

Oxidative stress can be defined as an increase above physiological values in the intracellular concentrations of reactive oxygen species (ROS) that include nitric oxide (NO), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), peroxyl radical ($ROO^{\bullet}$), and singlet oxygen (O_2). ROS factors are highly reactive due to the presence of one or more unpaired electrons in atomic or molecular orbitals. As a result of normal cellular respiration, the defence against oxidative stress is available to all cells via a series of enzymatic (e.g. catalase, superoxide dismutase (SOD)) and non-enzymatic (e.g. ascorbate, glutathione, alpha-tocopherol) antioxidants. When cell function is compromised by an insult or a depletion of energy levels, for example mechanical compression, ischaemia or glutamate excitotoxicity, the system becomes overloaded with damaging ROS that react with DNA, proteins and lipids.

Evidence that support the involvement of oxidative stress in glaucoma pathophysiology is strong. In glaucoma patients, analysis of blood and aqueous humour have demonstrated a markedly elevated levels of SOD and glutathione in aqueous (Ferreira *et al.* 2004), a significantly higher level of lipid peroxidation products (Birich *et al.* 1986), and low levels of glutathione in serum (Gherghel *et al.* 2005).

O_2^- is considered the "primary" ROS and can further interact with other molecules to generate "secondary" ROS (Valko *et al.* 2005). The production of O_2^- occurs mostly within the mitochondria (Cadenas & Sies 1998), and is formed by reduction of oxygen and mediated by NADH oxidases and xanthine oxidase or non-enzymatically by the semi-ubiquinone compound of the mitochondrial electron transport chain (Valko *et al.* 2007). O_2^- is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide and oxygen. NO is generated in biological tissues from arginine by specific nitric oxide synthases (NOSs), such as neuronal (NOS1), endothelial (NOS3) and inducible (NOS3) that is not found in normal physiological conditions. NO, an abundant reactive radical, acts as a potent physiological vasodilator, molecular messenger in the CNS as well as playing a role in defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi *et al.* 1999).

In the eye, there are three sites that are potentially susceptible to oxidative stress and subsequent glaucoma development: the TM, the ONH and the retina. In the retina, the expression of both types of SOD, cytosolic SOD1 and mitochondrial SOD2, has been found to be located in in the GCL and IPL in rodent retina (Oguni *et al.* 1995). In the ONH, the expression of NOS2, and up

regulation of NOS1 and NOS3 has been detected in astrocytes and microglia of patients with POAG and in experimental glaucomatous rat models (Liu & Neufeld 2000, Liu & Neufeld 2001, Neufeld *et al.* 1997, Pang *et al.* 2005, Shareef *et al.* 1999).

Glutamate excitotoxicity-induced oxidative stress has been linked to mitochondrial dysfunction in retinal ischemia and glaucomatous optic neuropathy. The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. Growing evidence indicates that glutamate excitotoxicity and/or oxidative stress is associated with mitochondrial DNA damage-related mitochondrial dysfunction in retinal neurodegeneration (Chan *et al.* 2012, Jarrett *et al.* 2008).

Neuroinflammation

The term neuroinflammation defines an inflammatory response organized within the nervous system, which arises following exposure to a trigger. In the retina and the brain, such a response has been documented after ischaemia (Barone *et al.* 1997, Yoneda *et al.* 2001), trauma (Knoblach *et al.* 1999) or excitotoxic assault (De Bock *et al.* 1996). Evidence supporting the contribution of cytokines, such as tumour necrosis factor–α (TNF-α) or interleukin-β (IL-1β) to neurotoxicity is considerable. An increase in the level of IL-1β mRNA expression after ischaemia has been noticed in the brain and the retina of rats (Hangai *et al.* 1995, Minami *et al.* 1992). An increased expression of immunoreactive IL-1β protein in both high IOP-induced retinal ischaemia/reperfusion and retinal cell culture excitotoxic rat models compared with normal retina has been shown to be reduced by administering the anti-IL-

1b antibody (Yoneda *et al.* 2001). Tezel and colleagues (2001) documented an increased expression of protein and mRNA of TNF-α and TNF-α receptor-1 in the retina of human glaucomatous eyes compared with control eyes of age-matched normal donors (Tezel *et al.* 2001). Furthermore, Balaiya and colleagues (2011) have reported a higher level of TNF-α expression in human aqueous obtained from patients with manifest glaucoma compared with disease free individuals (Balaiya *et al.* 2011).

Current glaucoma treatment principles

All currently available, clinically accepted, treatment strategies, both medical and surgical, are aimed at lowering IOP. Topical medical treatment has become the most commonly used initial therapeutic option followed by laser trabeculoplasty as an alternative. Therapeutic options target either the ciliary body to reduce aqueous production or the aqueous outflow pathways to stimulate aqueous drainage from the eye. At present, commonly used oculo-hypotensive medications fall into four categories: prostaglandin analogues, alpha2-adrenoceptor agonists, beta-adrenoceptor blockers, and carbonic anhydrase inhibitors. Prostaglandin analogues lower IOP by increasing uveoscleral outflow and have fewer systemic side effects than alpha2-agonists or beta-blockers. Alpha2-agonists and beta-blockers are effective at lowering IOP by reducing aqueous humour production via inhibition of adenylate-cyclase. In addition, alpha2-agonists have also been shown to increase uveoscleral outflow (Toris *et al.* 1999). Carbonic anhydrase inhibitors cause a reduction of aqueous production by the ciliary epithelium through blockage of carbonic anhydrase isoenzyme.

Laser trabeculoplasty is a therapeutic method designed to increase the outflow of the aqueous through the TM, either by stretching the TM (argon laser trabeculoplasty) or by stimulating TM cell function (selective laser trabeculoplasty).

Surgery is usually performed when topical or laser treatments have failed to control IOP sufficiently, there being a variety of options including penetrating (eg trabeculectomy) or non-penetrating filtration procedures or those involving the implantation of a drainage stent or tube.

Cyclo-ablative procedure (eg cyclodiode) reduces IOP by destruction of ciliary epithelium and is usually performed in advanced stages of glaucoma because of the potential risk of complications.

In the majority of cases, adequate reduction of IOP slows down the rate of glaucomatous changes. However, some patients still show evidence of significant glaucomatous progression despite a seemingly adequate reduction of IOP (Collaborative Normal Tension Glaucoma Study 1998, Heijl *et al.* 2002). Since reduction of IOP fails to halt disease progression in a proportion of patients and due to the fact that all current therapeutic strategies have their downsides, there has been a desire for alternative therapeutic strategies, such as neuroprotection.

There is a possibility that some currently available topical oculo-hypotensive agents may exert a direct neuroprotective effect that is different to the indirect protective effect of the reduced IOP. For example, the potential for direct

neuroprotective qualities of alpha2-agonists is supported by the presence of alpha2-adrenergic receptors not only in the non-pigmented ciliary epithelium, but also in RGC axons, dendrites, glia and photoreceptors (Woldemussie *et al.* 2007). Potential mechanisms for a neuroprotective action of brimonidine, a selective alpha2-agonist, include inhibition of glutamate excitotoxicity-induced oxidative stress (Lee *et al.* 2012), upregulation of BDNF expression (Gao *et al.* 2002), regulation of cytosolic Ca^{2+} signalling (Dong *et al.* 2007) and modulation of glutamate N-methyl-D-aspartate (NMDA) receptor function (Dong *et al.* 2008). In addition to the IOP-lowering effect of beta-blockers, it has been shown that betaxolol promotes rat retinal neurone survival after either ischaemic (Wood *et al.* 2003) or excitotoxic (Osborne *et al.* 1999) insults and levobetaxolol up-regulates BDNF expression in the retina (Wood *et al.* 2001). Prostaglandin analogues are powerful IOP-lowering agents that have also been shown to have direct neuroprotective properties. For example, it has been demonstrated that rat RGCs could be protected from apoptosis by latanoprost both *in vitro* and *in vivo* (Kanamori *et al.* 2009, Nakanishi *et al.* 2006, Zheng *et al.* 2011).

Even though there is scattered evidence for neuroprotective properties of some currently available topical treatments, the lack of large randomised studies in patients with glaucoma highlights the need for further research to identify new agents that protect undamaged and rescue dying RGCs in glaucoma. In the future, early identification of patients at high risk of developing glaucoma and initiation of novel neuroprotective treatments may have the potential to delay

the onset of detectable optic nerve damage and reduce the burden of glaucomatous visual loss.

Animal models used in glaucoma research

Successful research into any disease of humans is heavily dependent on the availability of laboratory research models that closely resemble human disease pathophysiology. Over many decades of research into glaucoma pathophysiology and treatment, many *in vivo* animal models have been developed, including large animals such as non-human primates (Dreyer *et al.* 1996, Quigley *et al.* 1995), cows and sheep (Gerometta *et al.* 2010, Tektas *et al.* 2010), horses (Whigham *et al.* 1999), dogs and cats (Brooks 1990, Brooks *et al.* 1997, Watanabe *et al.* 2003), pigs (Ruiz-Ederra *et al.* 2005), rabbits (Gherezghiher *et al.* 1986), and birds (De Kater *et al.* 1986, Kinnear *et al.* 1974), as well as small animals, such as mice (Buckingham *et al.* 2008, Joly *et al.* 2008, Pelzel *et al.* 2010, Senatorov *et al.* 2006), rats (Johnson *et al.* 2010, Leibinger *et al.* 2009, Sawaguchi *et al.* 2005) and zebrafish (Nagashima *et al.* 2011, Nishimura *et al.* 2014, Veth *et al.* 2011). Although a wide variety of animal species is being used in glaucoma research, only non-human primates closely resemble the anatomy and physiology of the human retina and the ON. In primate models of POAG, an increase in IOP has been achieved by mechanical obstruction of the aqueous outflow by means of argon laser photocoagulation of the TM (Burgoyne *et al.* 2004, Gaasterland & Kupfer 1974, He *et al.* 2014, May *et al.* 1997), latex microspheres injection into the anterior chamber (Weber & Zelenak 2001), or injection of autologous red

blood cells (Quigley *et al.* 1995) in order to study functional and anatomical changes in the retina and the ON. However, there are several limitations to the wide use of non-human primates in research, mainly animal accessibility and cost, as well as ethical aspects of using primates in research (Weinreb & Lindsey 2005).

Other non-primate small animal models, despite having differences in anatomy and physiology to humans, are extensively used because of the practicality and the reduced cost of purchase and maintenance compared to that of primates, the availability of large numbers for multiple repeats, and a possibility of genetic manipulations to study pathophysiology of the disease. A number of rat, mouse and rabbit *in vivo* models have been developed to study the effect of elevated IOP on the ON and RGC degeneration using cauterization or ligation of episcleral veins (Garcia-Valenzuela *et al.* 1995, Shareef *et al.* 1995, Yu *et al.* 2006b), and laser photocoagulation of the ciliary body or the TM (Gherezghiher *et al.* 1986, Gross *et al.* 2003, Levkovitch-Verbin *et al.* 2002b) and microbead injection into the anterior chamber (Sappington *et al.* 2010). Gene manipulations in order to create transgenic models to study normal tension and pigmentary glaucoma have also been used in mice (Anderson *et al.* 2002, Buckingham *et al.* 2008, Harada *et al.* 2007). For example, genetically modified mice deficient in the glutamate transporter gene *GLAST* have been created to study RGC and ON degeneration in a NTG model (Harada *et al.* 2007). A congenital model of glaucomatous RGC degeneration is also available in DBA/2J mice that develop persistent elevation of IOP in 6 to 8 months after birth. The mechanism of glaucoma development in DBA/2J mice

is different to POAG in humans and involves initial atrophy of the iris stroma with subsequent obstruction of the drainage angle due to inflammatory responses and subsequent pigment dispersion in the anterior chamber of the eye (Anderson *et al.* 2002, Chang *et al.* 1999). However, the progression of the RGC degeneration with age in DBA/2J mice is similar to glaucoma in humans (Anderson *et al.* 2002, John *et al.* 1998), making DBA/2J mice an attractive model to study glaucomatous RGC degeneration (Buckingham *et al.* 2008). A rat autoimmune model of glaucoma has also been developed and used to study the role of heat shock proteins (HSPs) in IOP-independent RGC degeneration and axonal loss by means of immunization of rats with HSP (Wax *et al.* 2008).

Despite the clear advantage of cost when compared to non-primates, there are numerous disadvantages of using small animal models in glaucoma research. Firstly, there are differences in the anatomy of the retina, retinal vasculature and the ON between experimental animal models and humans. For example, the ON of rodents lacks the LC and the retina has no macula. In rabbits, the retina is a relatively avascular structure, receiving its nutrition from the underlying choroid. Secondly, there are species differences that are particularly important when attempts are made to extrapolate the findings from rodents to humans. The classical example would be the wide use of albino rabbits in toxicology studies on topical glaucoma treatments due to known high sensitivity of albino eyes to ocular irritation. However, because the eyes of this species are non-pigmented and the tear production is known to be significantly lower than in humans (Rubin & Weisse 1992), the response to medications can be significantly more or less severe than in pigmented eyes. Another example

is when the use of dogs as a model for glutamate excitotoxicity research in relation to glaucoma had failed to demonstrate high levels of glutamate in vitreous (Kallberg *et al.* 2007), even though mechanism of congenital glaucoma in dogs is known to be caused by iridocorneal angle abnormality similar to humans. Moreover, mechanisms of high IOP in rodents are also different to humans. The failure of aqueous drainage in rodents is a result of persistent pupillary membrane causing pupil-block glaucoma or from peripheral anterior synechiae in uveitis (Kern 1997). On a cellular level across species, there is a difference in sensitivity to different retinal cell markers. For example, it has been shown that a AII subtype of amacrine cells in the rat retina is immunoreactive for parvalbumin, but not in the primate retina (Wassle *et al.* 1993). Conversely, another retinal cell marker calretinin labels amacrine cells in the primate retina (Wassle *et al.* 1995), but not in the rat retina. It can be followed from the above examples that the results obtained from small animal models cannot be transferred automatically to humans. Thirdly, mechanical interventions and genetic manipulations on rodents are unavoidably causing changes to other structures of the eye. Microophthalmia, for example, is a known problem in laboratory genetically modified animals (Lee 1989). Finally, there are ethical issues that have to be seriously considered when animals are being used in research, including repeated manipulations under general anaesthesia and animal handling if they are uncooperative. In fact, in some studies animals were kept alive for up to 2 weeks after optic nerve trans-sections before harvesting their retinas for analysis (Pelzel *et al.* 2010, Watanabe *et al.* 2003).

Although *in vivo* animal models can give insight into the pathophysiological changes associated with glaucoma, the development of *in vitro* models, such as retinal tissue and cell cultures, has allowed a better understanding of changes on a cellular level. Because *in vitro* models are maintained under controlled conditions, the response to alterations in their environment can also be studied, for example a neurotrophic effect of individual growth factors (Foxton *et al.* 2013) or an inhibition of a specific enzymatic pathway (Alonzi *et al.* 2001). Culture medium can also be used as a source of products released by cultured cells in order to investigate their secretory function (Johnson *et al.* 2014). Moreover, a benefit of direct visualization of the experiment allows a close monitoring of changes in cell morphology. Cell cultures can be used as a mixture of several cell types or a purified cell culture. Most of the ocular cell types have been cultured, including RGCs (Dun *et al.* 2007, Otori *et al.* 2003, Ozawa *et al.* 2013), trabecular TM cells (He *et al.* 2012), retinal astrocytes (Lukas & Wang 2012), the Müller cells (Von Toerne *et al.* 2014), and the RPE (Hettich *et al.* 2014, Stramm *et al.* 1983). Dissociated RGC cultures have, for example, been used to investigate the regeneration potential of retinal cells (Leibinger *et al.* 2009), to study oxidative stress as a mechanism of RGC death (Ozawa *et al.* 2013, Schlieve *et al.* 2006) and to test potential neuroprotective agents (Foxton *et al.* 2013, Otori *et al.* 2003). The main disadvantages of the retinal cell culture are the technical difficulties with obtaining sufficient numbers of cells for research and a potential loss of the cell line phenotype over the culture period. Recently, there have been several reports raising concerns related to the main cell line (RGC-5) used in the RGC research. The

RGC-5 cell line has originally been reported as a purified rat RGC culture via demonstration of characteristic RGC gene expression, including *Thy1* and *Brn3c*, and was promoted as a model for glutamate excitotoxicity and growth factors withdrawal studies (Krishnamoorthy *et al.* 2001). Since then the RGC-5 cell line was extensively used in glaucoma research, for example, as a model to study oxidative stress (Guo *et al.* 2013, Harper *et al.* 2009, Maher & Hanneken 2005), glutamate excitotoxicity (Aoun *et al.* 2003, Dun *et al.* 2007, Schultheiss *et al.* 2014) and also neuroprotective effects of various factors (Harper *et al.* 2009, Kanamoto *et al.* 2011, Schultheiss *et al.* 2013) However, recently the nature and phenotype of the RGC-5 cell line have been scrutinised (Krishnamoorthy *et al.* 2013, Van Bergen *et al.* 2009). The recent data suggest that the RGC-5 cell line represents a lineage of mouse neuronal precursor cells (Krishnamoorthy *et al.* 2013), that express a number of markers that are not specific to RGCs (Van Bergen *et al.* 2009).

Organotypic retinal culture

Animal organotypic retinal culture

To investigate the fundamental processes of RGC degeneration and test potential neuroprotective agents, several *in vitro* animal models have been developed. Rabbit, rat and mouse retinal explants or organotypic retinal cultures (ORCs) have been successfully used in research for several decades (Ames *et al.* 1992, Lucas & Trowell 1958). Preservation of the original tissue architecture and unique connections between neurons, and between neurons and glial cells, provides an opportunity to study cellular interactions in a

controlled environment. For example, ORCs have been used to study the response to light and retinal cell receptive fields (Koizumi *et al.* 2007), the spontaneous synaptic retinal cell activity (Perez-Leon *et al.* 2003), mechanisms involved in the apoptosis of RGCs (Beier *et al.* 2006, Manabe *et al.* 2002, McKernan *et al.* 2006, Zhang *et al.* 2008), and the effect of glutamate excitotoxicity (Haberecht *et al.* 1997, Xin *et al.* 2007), as well as the role of MSCs and MSC derived growth factors in neuroprotection (Bull *et al.* 2011, Johnson *et al.* 2014). It has also been shown that rodent ORCs can be cultured for an extended period (Caffe *et al.* 2001, Johnson & Martin 2008). Johnson and Martin (2008), for example, have demonstrated that rat organotypic retinal explants are viable for up to 17 days in culture and can be used as a model for studying the neuroprotective effect of mesenchymal stem cells (MSCs) on RGCs.

Human organotypic retinal culture (HORC)

The Norwich Glaucoma Research Group recently developed a method for the culture of human retinal explants. Niyadurupola and colleagues (2011) demonstrated that human organotypic retinal culture (HORC) was a useful model to investigate RGC death. It was demonstrated that gross morphology and retinal architecture of HORCs remained stable for at least 96 hours in culture, with a less than 10% reduction in RGC count. RGC markers have been used to assess RGC survival when known glaucomatous stresses have been applied, such as NMDA receptor activation and simulated ischaemia (oxygen/glucose deprivation (OGD)) (Niyadurupola *et al.* 2011). Using HORCs, Niyadurupola and colleagues (2011, 2013) have demonstrated that

human retinal tissue culture can be used as a useful model for investigation of complex mechanisms involving more than one retinal cell types. For example, the authors demonstrated the involvement of the $P2X_7$ receptor ($P2X_7R$) activation in RGC loss as a result of OGD (Niyadurupola *et al.* 2013) indicating a potential role of ATP-mediated excitotoxicity in glaucomatous neurodegeneration.

Additional work has been carried out on human retinal explants by other groups (Carr *et al.* 2009, Carter & Dick 2003, Mayer *et al.* 2005), but none of these studies looked at outcomes other than overall retinal structure. Adult human retinal explants, for example, have been used to investigate processes involved in the activation of microglia (Balasubramaniam *et al.* 2009, Carter & Dick 2003), to demonstrate degenerative changes in photoreceptors and interneuron cells morphology with time (Fernandez-Bueno *et al.* 2012), to demonstrate the presence of neural progenitor cells in the retina (Mayer *et al.* 2005), to investigate the potential of retinal cell out-growth from explants (Hopkins & Bunge 1991), and characterize retinal explants as a model for retinal angiogenesis (Knott *et al.* 1999). The longest period for the human retina tissue to be cultured has been reported by Kim and Takahashi in 1988, where the authors demonstrated by means of light and electron microscopy that retinal explants appeared to be alive for up to 4 months (Kim & Takahashi 1988), however there was no other evidence demonstrated to support this conclusion.

It can be followed from the above studies that Niyadurupola and colleagues (2011, 2013) were the first to characterize and use the HORC as an *in vitro*

model for investigating mechanisms involved in glutamate excitotoxicity and oxygen glucose deprivation in humans. However, it is still unclear whether or not HORCs can be used as a viable model for investigating effects of potential neuroprotective agents for longer than 3 days. The further development of an *in vitro* human retinal model could be of great benefit in research to understand the processes involved in glaucomatous neurodegeration over time, and in the development of new strategies for neuroprotection.

Aims of the thesis

The main aim of the work presented in this thesis was to investigate whether it is feasible to use HORCs as a long-term model of RGC degeneration. The second aim was to identify specific culture conditions that can potentially enhance the survival of RGCs in long-term HORCs. The potential neurotrophic effect of MSC derived growth factors on RGC survival was investigated, specifically leukaemia inhibitory factor (LIF), platelet derived growth factors (PDGFs) and vascular endothelial growth factor (VEGF). The effect of epigenetic regulation using the histone deacetylase inhibitor trichostatin A (TSA) on expression of RGC-specific markers in the HORC was also investigated.

CHAPTER 2

MATERIALS AND METHODS

Human donor tissue

Human donor eyes were obtained from the East Anglian Eye Bank (Norfolk and Norwich University Hospital, UK) within 24 hours *post mortem*. Information regarding past medical and ophthalmic history, and the age of the donors was recorded. Eyes with known retinal pathology were excluded from further tissue processing. The age range of the donors was between 60 and 87 years, with the average age being 73 years. The corneas were harvested for corneal transplantation at the East Anglian Eye Bank and the remaining parts of the eye were transported to the University of East Anglia in Eagle's minimum essential medium (EMEM) (Sigma Aldrich, Poole, UK) supplemented with 50µg/ml gentamycin (Sigma-Aldrich, Poole, UK) and 10µl/ml antibiotic/antimycotic solution, consisting of 10,000units/ml penicillin G and 10,000µg/ml streptomycin sulphate and 25µg/ml amphotericin B (Gibco Invitrogen, Paisley, UK).

The dissection technique

All procedures were performed under sterile conditions. Since the cornea had already been removed from the eye (figure 7A), the iris, the ciliary body and the lens were removed with scissors. An upper ring of the scleral cup was dissected to visualise the peripheral area of the retina (figure 7B). The neural retina was then carefully detached from the retinal pigment epithelium (figure

8C). One cut at the optic nerve head allowed separation of the retina from the scleral cup and underlying pigment epithelium ensuring minimal damage to the tissue (figure 7D). Linear cuts were made at the retinal edges and, if necessary, the vitreous was carefully removed with scissors to obtain flat positioning of the retina (figure 7E). A 4mm diameter micro-dissecting trephine (Biomedical Research Instruments, USA) was used to take five sections from the paramacular region of the retina (figure 7F). The sections were immediately placed into warmed serum-free Dulbecco's modified Eagle's medium (DMEM)/HamF12 (Gibco Invitrogen, Paisley, UK), supplemented with 50µg/ml gentamycin (Sigma-Aldrich, Poole, UK) or 100U/ml penicillin and 100µg/ml streptomycin (Gibco Invitrogen, Paisley, UK) and 2mM L-glutamine (Gibco Invitrogen, Paisley, UK). Initially, all five explants were placed in one dish to allow randomization of samples for the subsequent experiment. The HORCs were incubated at 35°C in a humidified atmosphere of 95% air/5% CO_2.

The template for HORCs positioning

The dissection of HORCs was performed as has been previously described (Niyadurupola *et al.* 2011) with further modifications. To ensure a consistent location of HORCs obtained from the paramacular region of the retina, a template was developed to aid the dissection (figure 7E). First of all, a 4mm circle from the area of the *fovea* was removed aiming for the transparent spot of the *foveola* (a yellow circle) to be as central as possible. All five paramacular circles were equally positioned from the central reference circle in relation to the optic nerve location.

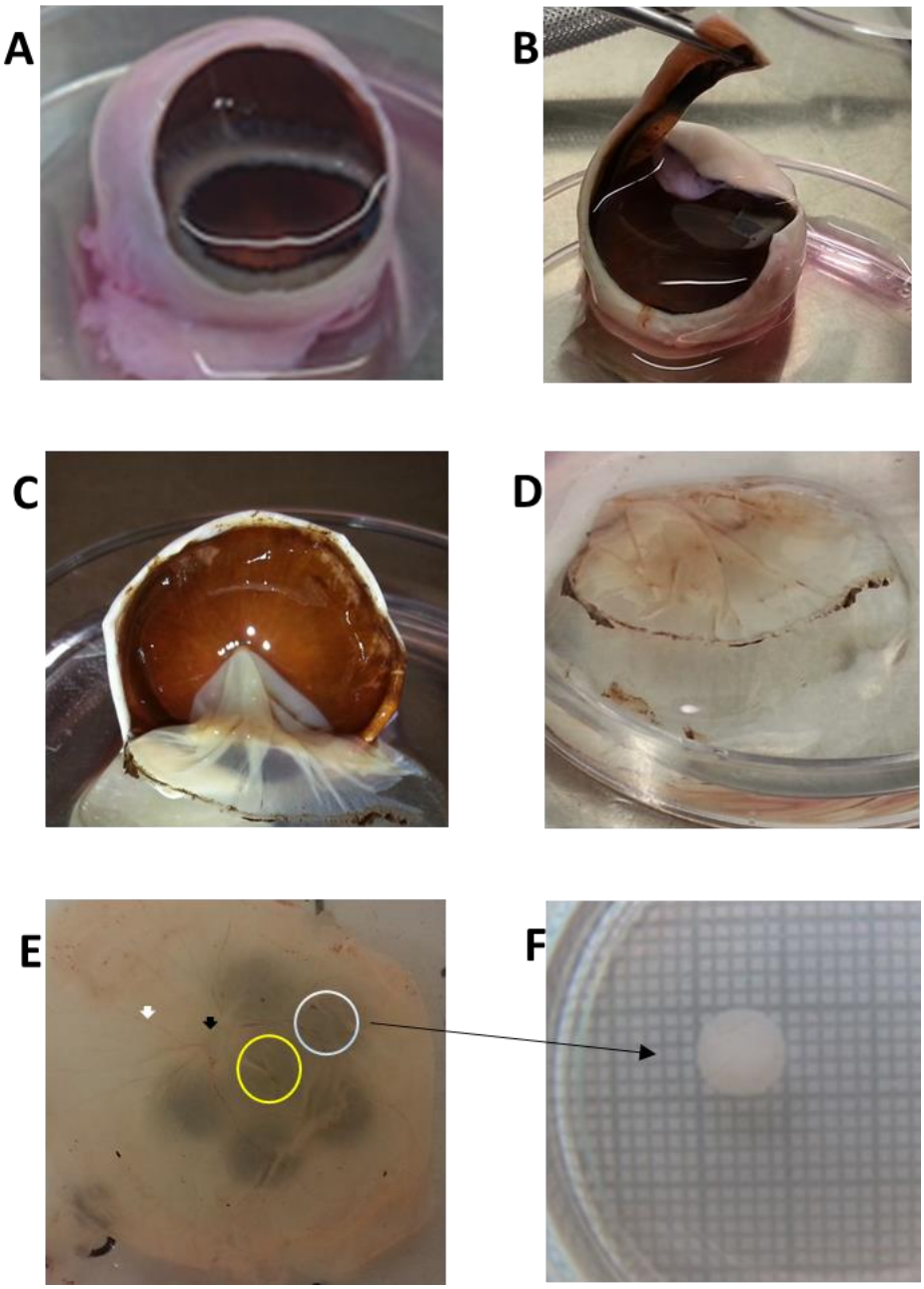


Figure 7: Representative images of the retinal tissue dissection. A) an eye globe with removed cornea, B) upper scleral rim dissection, C) the retina is attached at the ON, D) the free floating retina with vitreous is still attached, E) the flattened retina with template positioned beneath the dish, F) a free floating HORC. A yellow circle= 4mm area around the *foveola*, white circle= positioning of the 4mm trephine according to the template, small black arrow=optic nerve area, white arrow=retinal vessels.

The long-term human organotypic retinal culture model

HORCs were cultured for up to 4 weeks in Dulbecco's modified Eagle's medium (DMEM)/HamF12 (Gibco Invitrogen, Paisley, UK) or Neurobasal medium supplemented with 2% B27 and 1% N2 (Gibco Invitrogen, Paisley, UK). Explants were retrieved at weekly intervals for analysis. DMEM/HamF12 was used with or without supplementation with 10% foetal calf serum (FCS) (Gibco Invitrogen, Paisley, UK). Antibiotics used in cultures were either gentamycin at a concentration of 50µg/ml or a combination of 100U/ml penicillin and 100µg/ml streptomycin (Gibco Invitrogen, Paisley, UK). Half of the medium was replaced with fresh medium twice weekly.

Mesenchymal stem cell derived growth factors

Mesenchymal stem cell derived growth factors, specifically leukaemia inhibitory factor (LIF) (Sigma-Aldrich, Poole, UK), platelet derived growth factors (PDGF-AA, PDGF-AB) (PeproTech, USA) and vascular endothelial growth factor (VEGF) (Gibco Invitrogen, Paisley, UK), were used to investigate the potential of these compounds to extend the survival of RGCs in long-term HORCs. Growth factors were added to serum-free (SF) DMEM/HamF12 to obtain the following working concentrations: LIF was used at concentrations of 10, 30 and 50ng/ml, whereas PDGF-AA, PDGF-AB and VEGF at 50ng/ml concentrations. Control samples were maintained in parallel with SF DMEM/HamF12 with added TWEEN®20 (Sigma-Aldrich, Poole, UK) as a vehicle control at concentrations equivalent to experiments. There was no medium changed over the time of experiment which lasted 1 week.

Histone deacetylase (HDAC) inhibitors

Histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) (Sigma-Aldrich, Poole, UK) was used in experiments intended to extend the expression of retinal cell mRNA in HORCs. TSA was added to SF DMEM/HamF12 to make concentrations of 0.1, 1 and 10µM. Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Poole, UK) was used as a solvent and it was, therefore, added to the medium of control HORCs as a vehicle control at concentrations equivalent to experiments. There was no medium change made during time of the experiment which lasted 1 week.

Immunohistochemistry

Tissue fixation and sectioning

Upon completion of the experiment, HORCs were fixed in 4% paraformaldehyde for 24 hours at 4°C. The following day, explants were transferred into 30% sucrose solution in phosphate buffered saline (PBS) for cryopreservation (Oxoid, Basingstoke, UK) for 24 hours. To prepare for sectioning, the samples were frozen on dry ice in optimal cutting temperature (OCT) medium (Sakura Finetek, Zoeterwoude, NL), and stored at -80˚C. Retinal slices of 13µm were cut from frozen block moulds using a Hacker Bright OTF 5040 cryostat (Bright Instruments, Huntingdon, UK). At least three non-consecutive slices were collected on 3-aminopropyl-triethoxyl saline (TESPA) coated glass slides (Sigma Aldrich, Poole, UK) ensuring that each slice was taken over 50µm apart from the previous cut. Slides were stored at -20°C if needed.

Immunolabelling

Slides with retinal sections were washed 3 times for 10 minutes in 0.1M PBS with gentle agitation to remove the OCT medium. To penetrate the cell membrane and block non-specific binding sites, the retinal sections were incubated in blocking solution containing 0.2% Triton-X-100 (Sigma Aldrich, Poole, UK) and 5% normal goat serum (Sigma Aldrich, Poole, UK) in 0.1M PBS for 90 minutes at room temperature. After removal of the blocking solution, the retinal slices were incubated with primary antibodies (Table 1) made up in blocking solution for 24 hours at 4°C. The following day, the retinal slices were washed 3 times for 10 minutes in 0.1M PBS with gentle agitation, followed by incubation for 2 hours in the dark at room temperature with secondary antibody diluted in blocking solution to the required concentration (Table 1). Negative controls were processed in parallel by omission of the primary antibody. Following the incubation, the slides were washed three times in 0.1M PBS and then counterstained with 4', 6-diamidino-2-phenyindole dilactate (DAPI) (Invitrogen, Paisley, UK) at concentration of 0.5µg/ml for 10 minutes at room temperature in the dark. The slides were again washed 3 times for 10 minutes in 0.1M PBS with gentle agitation. Each retinal slice was mounted with one drop of hydromount immunofluorescence medium (National Diagnostics Ltd, Hull, UK) and sealed with a coverslip (VWR International, Lutterworth, UK). The slides were placed in the dark at room temperature to dry and stored at 4°C in the dark.

Target	Clone/ Conjugate	Source	Company	Dilution
Primary antibodies				
Neuronal Nuclei (NeuN)	Monoclonal	Mouse	Chemicon International, Millipore, Watford, UK	1:200
Secondary antibodies				
Mouse IgG (H+L)	AlexaFluor 488	Goat	Invitrogen, Paisley, UK	1:1000
Rabbit IgG (H+L)	AlexaFluor 568	Goat	Invitrogen, Paisley, UK	1:1000

Table 1: Details of primary and secondary antibodies used in immunohistochemistry.

Retinal slices were imaged using a wide-field Zeiss Axiovert 200M fluorescent microscope (Zeiss, Welwyn Garden City, UK). Zeiss Axiovision 4.8 software was used to analyze images.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

Apoptotic DNA damage in the HORCs was detected by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay. The DeadEnd™ Fluorometric TUNEL System (Promega, Southampton, UK) labels the fragmented (nicked) DNA by catalytically

incorporating fluorescein-12-dUTP at 3´-OH DNA ends using recombinant Terminal Deoxynucleotidyl Transferase enzyme (rTdT). The kit was used according to the manufacturer's instructions.

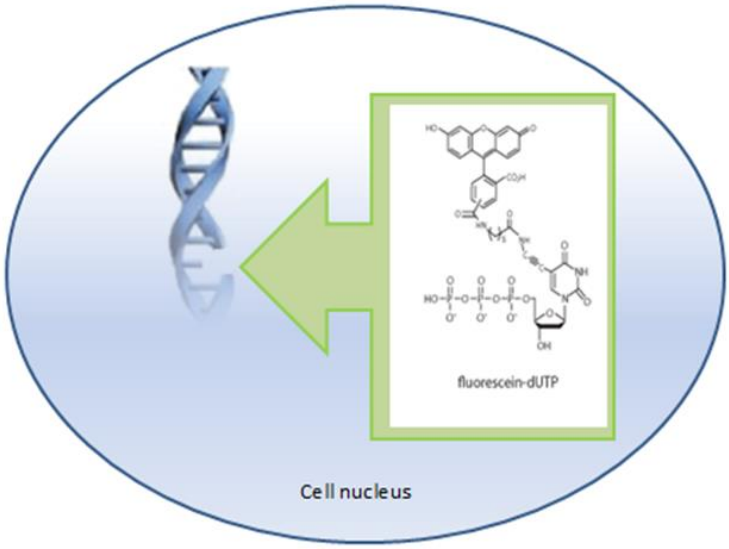


Figure 8: Schematic representation of modified dUTP incorporation by the enzyme terminal deoxynucleotidyl transferase (TdT) at the 3'-OH ends of fragmented DNA.

The slides were washed and incubated with the required primary antibody overnight, followed by 3 further washes as described in the previous section. To remove excess fluid, the slides were carefully tapped on a paper towel and then covered with "equilibrium buffer" at room temperature for 10 minutes. The "equilibrium buffer" was gently removed and each retinal slice was then covered with a mix of 45µl equilibrium buffer, 5µl nucleotide mix and 1µl rTdT enzyme for 60 minutes at 35˚C in a humidified chamber in the dark. The reaction was terminated by immersing the slides in standard saline citrate

(SSC) (3M NaCl, 340mM $NaH_2C6H_5O_7$), diluted from X20 stock to X2. The slides were again washed 3 times for 10 minutes in 0.1M PBS with gentle agitation. Secondary antibody and all subsequent stages were performed as described in the previous section. Positive controls were treated with RNase-free DNase 1 (Qiagen, Crawley, UK) for 10 minutes at room temperature prior to the equilibrium buffer stage (figure 9). The images were taken by using a wide-field Zeiss Axiovert 200M fluorescent microscope (Zeiss, Welwyn Garden City, UK) and analysed with Zeiss Axiovision 4.8 software.

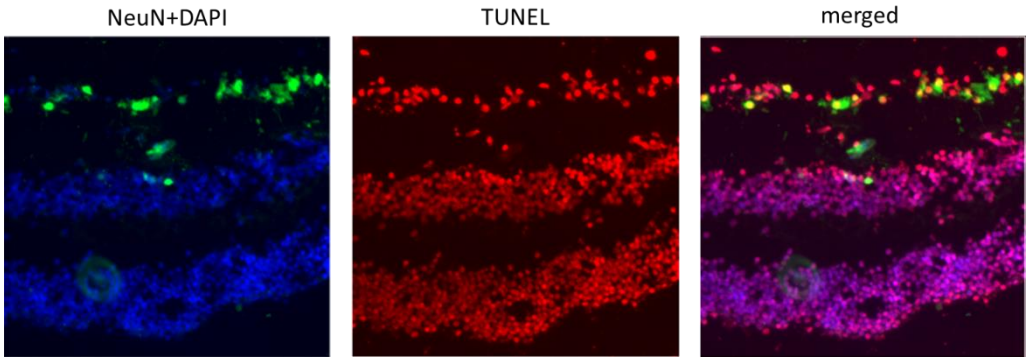


Figure 9: TUNEL positive control treated with RNase-free DNase 1. Cell nuclei were labelled with DAPI (blue); RGCs were labelled with NeuN (green); apoptotic cell nuclei were labelled with TUNEL (red).

Assessment of retinal ganglion cell loss

Quantification of RGCs in the HORC was performed by counting NeuN–labelled cells in the RGCL in at least 27x200µm areas per explant in a masked fashion. Each image was randomly coded prior to counting by a different member of the research group. The mean of NeuN-positive RGCs was calculated for each HORC. The number of apoptotic RGCs in the HORC was detected by use of TUNEL as described above. The proportion of TUNEL-

positive NeuN-labelled RGCs was calculated by counting the number of cells in the RGC layer that were stained positive with both NeuN and TUNEL.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Quantitative real-time polymerase chain reaction was used to measure the level of expression of specific mRNAs in HORCs. Total RNA was extracted and then reverse transcribed to synthesise complementary DNA (cDNA). The amount of the target cDNA in a sample was quantified by using the TaqMan® QRT-PCR method. Details of the methods are given below.

RNA extraction

RNA extraction was carried out using a column-based method using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Crawley, UK). After the experiment, HORCs were snap frozen in liquid nitrogen and stored at -80ºC. The tissue samples were placed into guanidine-thiocyanate-containing lysis buffer and homogenised by repeatedly passing them through a 20-gauge needle fitted to an RNase-free syringe at least eight times. The lysate was centrifuged at 13,000 rpm for 3 minutes to separate the supernatant which was mixed with 70% ethanol and transferred to the RNeasy Mini spin column. The column was centrifuged for 15 seconds at 13,000 rpm allowing the total RNA to bind to the membrane of the RNeasy spin column. Any contaminants were washed away with buffer RW1. DNA removal was achieved by adding RNase-free DNase 1 (Qiagen, Crawley, UK) to the column for 15 minutes. The DNase 1 was washed away with buffer RW1. The column

was then washed twice with ethanol containing buffer RPE, followed by a two-minute centrifugation at maximum speed to dry the spin column membrane, ensuring no carryover of ethanol during RNA elution. RNase-free water (50µl) was used to elute the total RNA. A volume of 30µl RNase-free water was used in the long-term experiments due to a low yield of total RNA at week 3 and 4. The quantity and the quality of total RNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The RNA was stored at -80 ˚C.

First strand complementary DNA (cDNA) synthesis

RNase free water (Qiagen, Crawley, UK) was used to dilute total RNA to obtain an equal amount of RNA in each sample. A volume of 10µl diluted RNA was mixed with 1µl random primers (Promega, Southampton, UK) and 1µl 10mM dNTP mix (containing dATP, dGTP, dTTP, dCTP) (Bioline, UK). Samples were then incubated at 65˚C for 5 minutes using a Peltier Thermal Cycler DNA engine (PTC-200, MJ Research, Minnesota, USA). The samples were briefly chilled on ice. A mixture of 4µl 5X First strand buffer (Invitrogen, CA, USA), 2µl 0.1M Dithiothreitol (DTT) (Invitrogen, CA, USA) and 1µl RNase inhibitor (Promega, Southampton, UK) was added to each sample. The samples were incubated at 25˚C for 2 minutes. 1µl of Superscript® II reverse transcriptase (Invitrogen, CA, USA) was then added to each sample. The samples were incubated at 25˚C for 10 minutes and then at 42 ˚C for 50 minutes. The reaction was inactivated by incubation at 70˚C for 15 minutes. Samples were stored at -20˚C.

TaqMan® QRT-PCR

TaqMan® QRT-PCR is based on the use of a dual-labelled oligonucleotide probes. The TaqMan® probe has a reporter dye located at the 5'end and a quencher dye at 3'end. The quencher absorbs fluorescence produced by the reporter dye and, therefore, no fluorescence is emitted in the intact probe. The signal is only produced after the reporter and quencher dyes are separated by enzymatic breakdown of the probe by the Taq polymerase. A polymerase chain reaction starts with the heating of the double stranded DNA to form a single strand. A specifically designed probe for the target nucleotide anneals to the 3' end of the target gene. The sequence extends via Taq polymerase enzyme, which releases the reporter from the probe to produce fluorescence. The intensity of fluorescence directly correlates with the amount of the target gene. The process is summarised in figure 10.

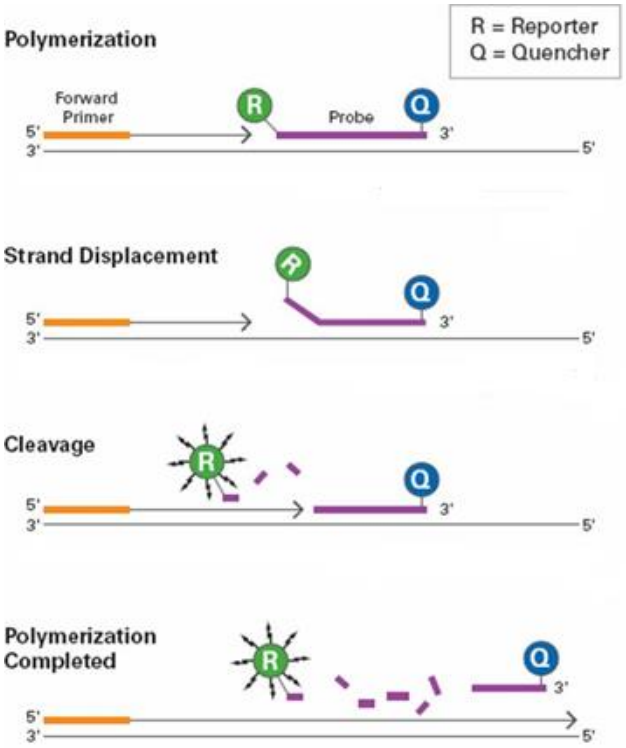


Figure 10: Schematic representation of the principle for the real-time polymerase chain reaction using TaqMan®. In the initial stage, primers and probes anneal to template DNA strand, followed by extension of the complementary sequence via Taq polymerase and displacement of the probe. Finally, the probe is broken and the quencher is released. Fluorescence is measured to quantify the amount of the target gene. Image adapted from http://www.appliedgene.com/realTimePCR_2.html.

The cDNA was diluted with nuclease free water (Promega, Madison, USA) to give 5ng total cDNA in 10µl. TaqMan® PCR Master Mix (Applied Biosystems, Warrington, UK) and the primers and probes (Table 2) were mixed to make a total volume of 25µl. The reaction set up for the PCR amplification was 50˚C for 2 minutes, 95˚C for 10 minutes, followed by 40 cycles consisting of 15 seconds at 95˚C and followed by 1 minute at 60˚C for each cycle. QRT-PCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystems, Warrington, UK).

Genes	Code	Company
Thy1	Hs00174816_ml	Applied Biosystems, Warrington, UK
ChAT	Hs00758143_m1	Applied Biosystems, Warrington, UK
POU4F1	Hs00366711_m1	Applied Biosystems, Warrington, UK
CALB	Hs00191821_m1	Applied Biosystems, Warrington, UK
RCVN	Hs00610056_m1	Applied Biosystems, Warrington, UK
Rbfox3	Hs00876928_m1	Applied Biosystems, Warrington, UK
HSPA1B	Hs01040501_sH	Applied Biosystems, Warrington, UK
Glul	Hs00365928_q1	Applied Biosystems, Warrington, UK
GLAST	Hs00188193_m1	Applied Biosystems, Warrington, UK
CYC1	Hs00357718_g1	Primer Design, Southampton, UK
TOP1	Hs00243257_m1	Primer Design, Southampton, UK

Table 2: TaqMan probes suppliers and code information.

For each sample the point at which the fluorescent signal becomes statistically above background, the threshold cycle (CT) value, was determined with the 7500 Fast System Software (Applied Biosystems, Warrington, UK). The standard curves were produced for each gene of interest and used for quantification of gene expression.

To eliminate variations in the gene expression due to differences in the overall amount of mRNA, the expression level of the gene of interest was normalized to that of two "housekeeping" genes. These genes were selected for their relatively stable level of expression in experiments carried out using HORCs (Niyadurupola *et al.* 2011). The normalising genes were topoisomerase 1 (*TOP1*) and cytochrome c 1 (*CYC1*).

Lactate Dehydrogenase (LDH) cytotoxicity detection assay

Cell death in the HORC was quantified by measuring lactate dehydrogenase (LDH) released into the culture medium (figure 11). LDH is an enzyme that is released when the integrity of a cell membrane is compromised. Extracellular LDH has been found to be both chemically and biologically stable and is present in all cells (Koh & Choi 1987).

The LDH test is based on the enzymatic reaction where LDH acts as a catalyst for lactate conversion to pyruvate. At the same time, tetrazolium salt receives the H^+ ion and becomes formazan which is a coloured product. The amount of colour formed by the appearance of formazan correlates directly with the amount of LDH released into the medium.

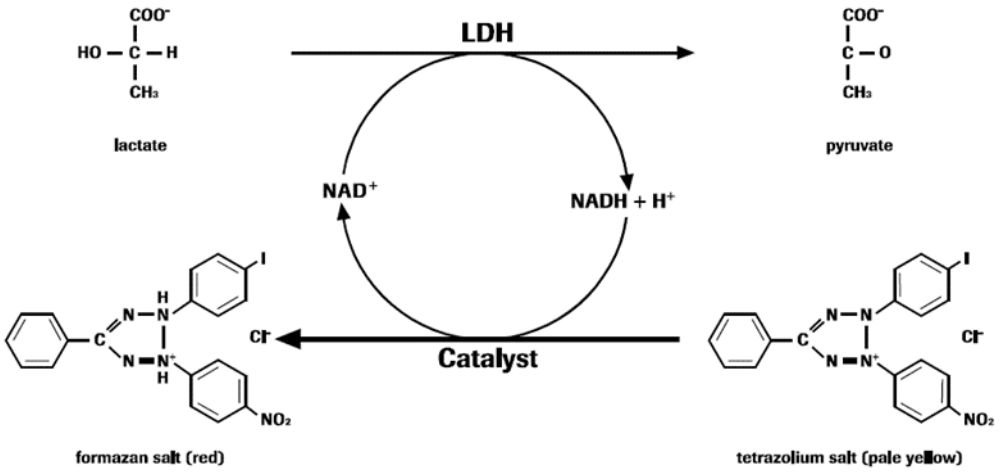


Figure 11: Enzymatic reaction catalysed by presence of LDH. Adapted from Roche manual.

For LDH analysis medium was collected from cultures and centrifuged at 13,000 rpm for 5 minutes to remove cell debris. Medium was then pipetted into a 96 well plate (100µl per well) in triplicate. Medium measured from long-term HORCs was diluted 1:1 with SF DMEM/HamF-12 medium in order to prevent signal saturation in case of a significant cell death level in the tissue rather than cell culture. SF DMEM/HamF-12 was used as a background control. An equal volume of LDH cytotoxicity detection solution was added to each well, which consisted of diaphorase/NAD+ mixture with iodotetrazolium chloride and sodium lactate, prepared according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The plate was incubated for 5 minutes in the dark at room temperature. Dye production was measured at 490nm using a 96 well

plate reader (Victor EG & G Wallace Instruments, Cambridge UK). After subtraction of background readings, LDH values were normalized to the control.

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Significance was determined using Student's t-test or one-way ANOVA with Dunnett's and Tukey's post hoc tests (GraphPad Prism; Graph-Pad Software Inc., La Jolla, Ca, USA). Values of $p \leq 0.05$ were considered to be statistically significant.

CHAPTER 3

RGC SURVIVAL AND DEVELOPMENT OF CULTURE CONDITIONS FOR A LONG-TERM HUMAN ORGANOTYPIC RETINAL CULTURE

Introduction

Human organotypic retinal cultures (HORCs) have been established as a useful experimental system for investigation of the mechanisms involved in RGC death, as well as for testing of potential neuroprotective compounds by modelling glaucomatous stress in the short-term, for example by NMDA receptor activation or simulated ischaemia (Niyadurupola et al. 2013, Niyadurupola et al. 2011). It has been demonstrated that HORCs preserved their appearance and retinal cell layer architecture for at least 96 hours when cultured in serum-free (SF) Dulbecco's modified Eagle's medium (DMEM)/HamF12 (Niyadurupola et al. 2011). The authors also tested the effect of Neurobasal medium supplemented with B27/N2 on RGC survival in HORCs, which had previously been shown to be the optimum medium for rat organotypic retinal cultures (Johnson & Martin 2008). There was no difference between tested culture conditions in the gross appearance and the timeline of the RGC mRNA loss in HORCs after 96 hours in culture. These findings led to a conclusion that SF DMEM/HamF12 was the most appropriate medium for culture of human retinal explants since its composition was known, whereas an exact compositions of B27 supplement was still commercially protected (Cressey 2009, Niyadurupola *et al.* 2011).

Although Niyadurupola's study was the most extensive in terms of characterising the human retina as an *in vitro* model for RGC neurodegeneration, there is a considerable body of research published by other study groups using human tissue to study mainly physiological changes within the retina. The earliest reported and the longest human retina culture to date is a study by Kim and Takahashi published in 1988. The authors have maintained the human retina in serum supplemented Eagle's minimum essential medium (EMEM) with added gentamycin and amphotericin B. The results of the study were documented by means of photomicrographs and electron microscopy indicating that retinal cultures can maintain their gross appearance and retain a considerable degree of retinal cell layer organization for a period as long as 4 months. The quality of the donor retinal tissue was reported as good or excellent in only half of the cases with a considerable degree of variation in a number of surviving cells in the retinal GCL (Kim & Takahashi 1988). Later on, another study looked at the potential of the human retina to be used as a model for retinal angiogenesis research (Knott *et al.* 1999). Adult human retinal explants were cultured in fibrin matrix in Glasgow's minimal essential medium (GMEM) with added growth factors, serum and without antibiotics for 14 days. The authors found the presence of an extensive vascular network within the retinal explants, as well as new vessel outgrowth into the fibrin matrix by day 10 (Knott *et al.* 1999). The findings of those studies support the potential of the human retinal explants to maintain their viability in a long-term culture. Other groups have used human retinal explants in various culture conditions to investigate the potential for retinal cell growth from explants

(Hopkins & Bunge 1991, Thanos & Thiel 1990), to understand mechanisms involved in activation of the retinal glia (Carter & Dick 2003), to demonstrate the presence of retinal progenitor cells (Mayer *et al.* 2005), to analyse the expression of retinoblastoma protein and to study retinal progenitor cells (Donovan & Dyer 2006), and to describe degenerative changes within the retina (Fernandez-Bueno *et al.* 2012). However, different culture conditions were used by different groups and none of these studies assessed the effect of culture conditions on overall retinal tissue viability. For example, Carter and Dick (2003) used the human retina to investigate processes involved in the activation of microglia by culturing human retinal explants in Roswell Park Memorial Institute (RPMI) medium supplemented with serum, penicillin and streptomycin for 72 hours (Carter & Dick 2003). Mayer and colleagues (2005) used DMEM/HamF12 supplemented with serum and multiple growth factors and without antibiotics to culture human retinal explants for 5 days in order to demonstrate the presence of neural progenitor cells in the adult human retina (Mayer *et al.* 2005). The most recent study by Fernandez-Bueno and colleagues (2012) characterised the human retina as a model for studying degenerative processes mainly in the outer layers of the retina, specifically photoreceptors, horizontal and bipolar cells, by maintaining human retinal explants in Neurobasal medium supplemented with B27 and serum, as well as a combination of penicillin/streptomycin and amphotericin B. The results of the study were rather limited (n=2), and that the study was based only on immunostaining and electron microscopy which demonstrated a gradual pyknosis of retinal cell nuclei and some vacuolization of retinal layers

associated with loss of the photoreceptor outer segments over a period of 9 days (Fernandez-Bueno *et al.* 2012). It appears that HORCs, characterized by Niyadurupola and colleagues (2011) as the model for RGC degeneration, is the only human model available to date for *in vitro* glaucoma treatment studies using human retina. However, HORCs were characterized only for 96 hours, limiting the use of HORCs for testing neuroprotective compounds beyond this time point.

There have been many more studies using organotypic retinal cultures from other species to study retinal physiology, for example in mouse (Caffe et al. 2001, Ferrer-Martin et al. 2014, Perez-Leon et al. 2003), rat (Feigenspan et al. 1993, Johnson & Martin 2008, Manabe et al. 2002), rabbit (Koizumi et al. 2007, Lye et al. 2007) and pig (Carr et al. 2009, Fernandez-Bueno et al. 2008). However, the most comprehensive study characterizing rat organotypic retinal cultures as a model for investigation of the retinal stem cell therapy for treatment of neurodegenerative disease was published by Johnson and Martin (2008). The authors showed that serum-free Neurobasal medium supplemented with B27/N2 and added penicillin/streptomycin was the condition of choice to maintain rat organotypic retinal explants for up to 17 days. Viability of retinal explants was demonstrated by means of photography, propidium iodine uptake and immunohistochemistry for neural and glial retinal cell markers, as well as immunolabelling with caspase-3 antibodies as a marker for apoptosis. An increase in the retinal explant degradation, a significant down regulation of retinal and glial cell markers together with a significant increase in a level of apoptosis were associated with supplementation of the medium with horse

serum rather than B27/N2 after 10 days in culture (Johnson & Martin 2008). The longest mammalian retinal culture that has been reported, to our knowledge, was a mouse retinal culture in serum-free R16 nutrient medium for up to 4 weeks (Caffe *et al.* 2001). The authors were aiming to prove that both adult and neonatal retinal tissues could be maintained in serum-free conditions for a prolonged period of time, however the effect of serum supplementation on tissue viability was not tested. Although viability of retinal explants was documented by RT-PCR and immunohistochemistry, interestingly, there was no statistical quantification of the results (Caffe *et al.* 2001). Viability of the retinal tissue *in vitro* has also been demonstrated by recording electrophysiological responses to light, that were within the normal range, from RGCs in rabbit retinal explants cultured in Ames' medium containing horse serum, N2 supplement, and penicillin/ streptomycin for up to 6 days (Koizumi *et al.* 2007). The presence of spontaneous synaptic activity in mouse retinal explants was also recorded after 14 days in culture using Hank's basal medium and Hank's BSS supplemented with horse serum (Perez-Leon *et al.* 2003). It is possible to suggest that serum might be beneficial, however recently another group demonstrated, in agreement with Johnson's findings, that an addition of horse serum in culture of adult mouse retinal explants caused significant alterations in the retinal tissue architecture after 10 days in culture (Ferrer-Martin *et al.* 2014), indicating a potential negative effect of serum on retinal cell viability in a long-term culture. Despite some inconsistencies, the findings of the above studies support the potential of retinal explants to be used as a long-term model for retinal neurodegeneration. The development of extended

human organotypic retinal cultures would be the most relevant model for neuroprotective studies of human neurodegenerative diseases.

The aim of the work described in this chapter was to investigate the long-term RGC survival in HORCs maintained for extended period (up to 4 weeks) and to establish culture conditions to prolong RGC survival. Two medium types were tested, DMEM/HamF12 (Niyadurupola *et al.* 2011) and Neurobasal medium (Johnson & Martin 2008), and the effect of serum supplementation was also assessed. In addition, due to lack of agreement in the choice of antibiotics for the retinal tissue culture and a considerable body of evidence regarding the toxic effect of gentamycin on the retina (Campochiaro & Conway 1991, Hancock *et al.* 2005, Kanter & Brucker 1995, Penha *et al.* 2010), it was also important to look at choice of antibiotic for HORCs.

Results

Time dependant changes of human retina explants over 4 weeks in culture

Initial experiments were set up using culture conditions determined by Niyadurupola and colleagues (2011) for a short-term culture of human retinal explants to examine whether the culture time could be extended under those conditions. HORCs were, therefore, cultured in SF DMEM/HamF12 medium with added gentamycin for 4 weeks. Because of the length of experiments, there was a regular twice weekly change of half of the medium, as described by Johnson and Martin (2008), in order to supplement the retinal tissue explant with fresh feeding substrates and dilute waste products. Out of 5 explants

obtained from the retinal paramacular region, one sample was fixed immediately either in 4% paraformaldehyde for immunohistochemistry or frozen in liquid nitrogen for QRT-PCR. Each subsequent week, one of the remaining explants was harvested in the same fashion for further processing. In order to document the gross appearance and size of HORCs over the culture period, photographs were taken at weekly intervals (figure 12). The size of explants was measured by taking a photograph of each explant with graph paper being placed under the dish. Each sample had 6 radial measurements taken. Each measurement was normalised to the size of the small square on the graph paper. Over the 4 week period, it can be seen that HORCs preserved overall appearance, tissue integrity and colour. There was a gradual decrease in the diameter of retinal explants (figure 13) that could be mainly explained by curling of tissue margins. An approximately 20% decrease was documented by week 2, followed by a further gradual reduction in size over the next two weeks, reaching approximately 75% of its original size by week 4 ($p<0.005$, $n=3$).

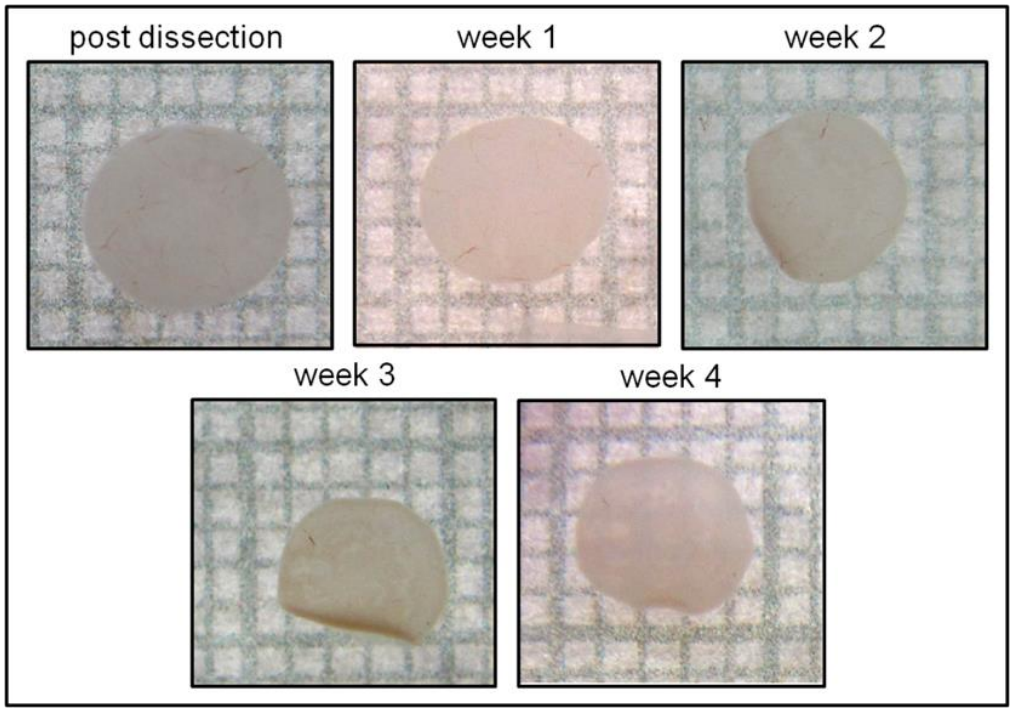


Figure 12: Photographs of HORCs in SF DMEM/HamF12 over the 4 week culture period. Each image was taken at weekly interval to document gross appearance and size of explants was measured over time.

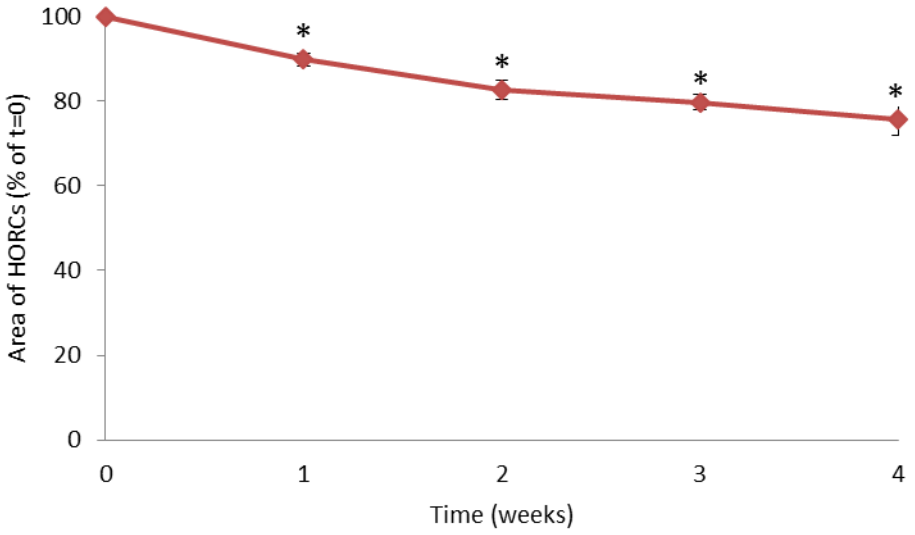


Figure 13: Change in diameter of HORCs with SF DMEM/HamF12 over 4 weeks in culture. Sample diameter was calculated by taking the mean of 6 radial measurements. Each measurement was normalized to the size of the small squire on graph paper (mean±SEM, n=3). The results are presented as % of t=0. Student's t-test was used, *$p<0.005$.

The next step was to examine the appearance of the retinal cell layer architecture at the cellular level by means of immunohistochemistry. As can be seen in figure 16, the laminar structure of all cell layers was well-preserved at each time point. The level of apoptosis within retinal explants was detected by immunolabelling of the sections with TUNEL. There were only a few apoptotic cells detected in t=0 sections. At week 1, the presence of apoptotic nuclei was mainly documented in the GCL and in the inner aspect of the INL indicating that RGCs, and possibly amacrine cells, are the first cell types to sustain DNA damage. Over time, there was a gradual increase in the number of TUNEL-positive nuclei in all cell layers. The most intensive uptake of TUNEL immunolabelling in all cell layers was documented after 4 weeks in culture (figure 14).

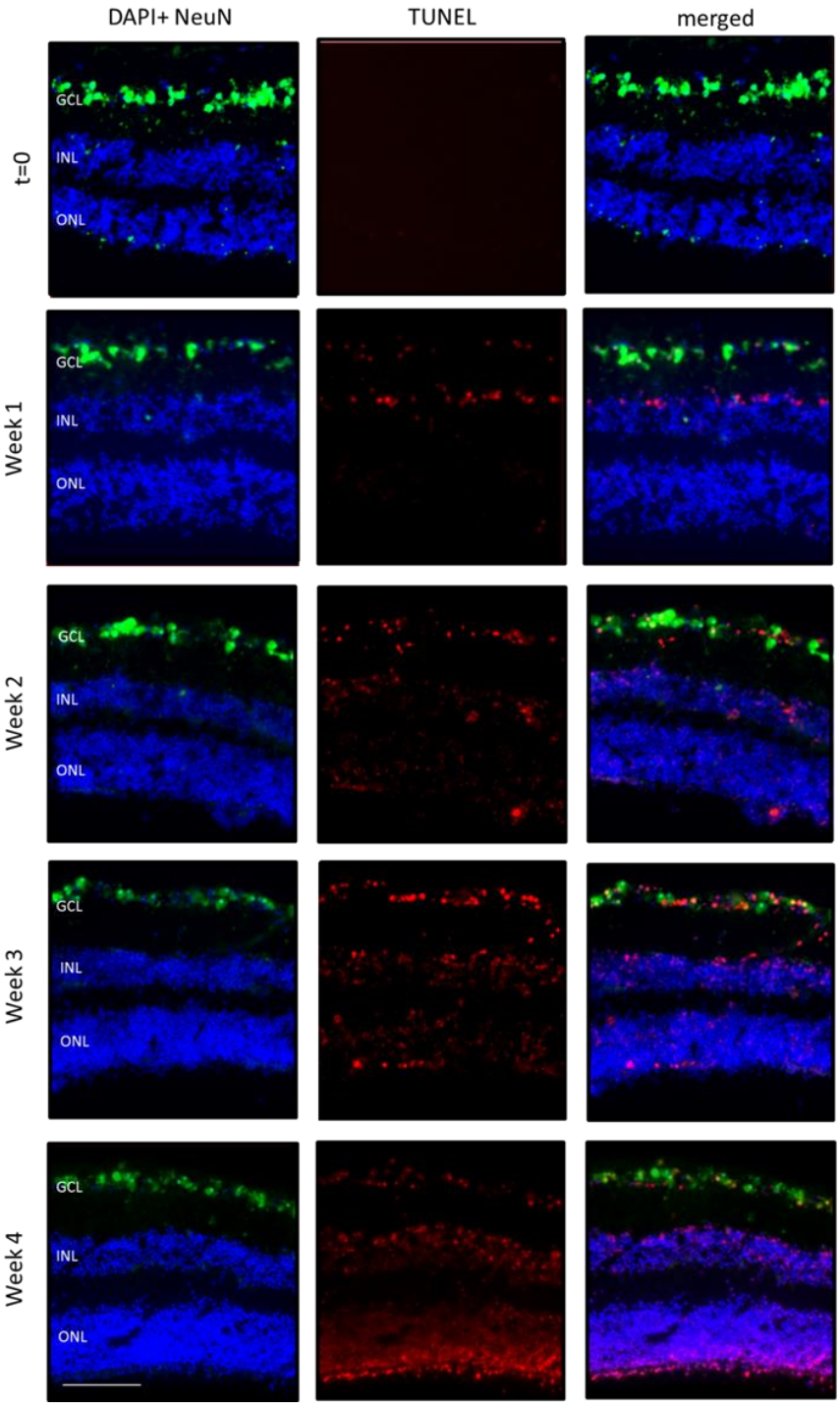


Figure 14: Representative images of HORC immunohistochemistry and TUNEL-labelling of HORCs in SF DMEM/HamF12 at each time point. NeuN (green) is a marker for RGCs; TUNEL (red) is a marker for apoptosis. Cell nuclei are stained with DAPI (blue). Scale bar, 100 µm.

In order to characterise long-term HORCs as a model of RGC degeneration, the loss of RGCs with time was estimated by counting NeuN-labelled cells in the GCL at each time point. The neuron specific protein, NeuN, is a sensitive and specific marker of neuronal somata (Wolf *et al.* 1996). NeuN antibodies have been used for RGC labelling in human, mouse and rat retinal explants (Buckingham *et al.* 2008, Diaz *et al.* 2005, Niyadurupola *et al.* 2011, Wolf *et al.* 1996). In HORCs, NeuN-labelled RGCs were mainly detected in the GCL. The presence of a few NeuN-labelled amacrine cells in the inner aspect of the INL of the retina was also noticed. In the literature, it has been documented that a subset of amacrine cells can also be NeuN positive (Wolf *et al.* 1996). It has been estimated, however, in a mouse model of RGC degeneration that the proportion of NeuN positive amacrine cells is age dependent and varies between 4 and 16% (Buckingham *et al.* 2008). In the human retina, there were a few cells labelled with NeuN in the INL, indicating that even if they are amacrine cells the labelling of these cells is not excessive. In fact, the labelling could also be explained by the presence of displaced RGCs in the INL. As can be seen in figure 15, there was a gradual loss of NeuN-labelled RGCs in the RGCL, which reached a statistically significant level of approximately 20% by week 2 ($p<0.005$, $n=6$) and remained at a relatively stable level between weeks 2 and 4 ($p<0.005$, $n=6$).

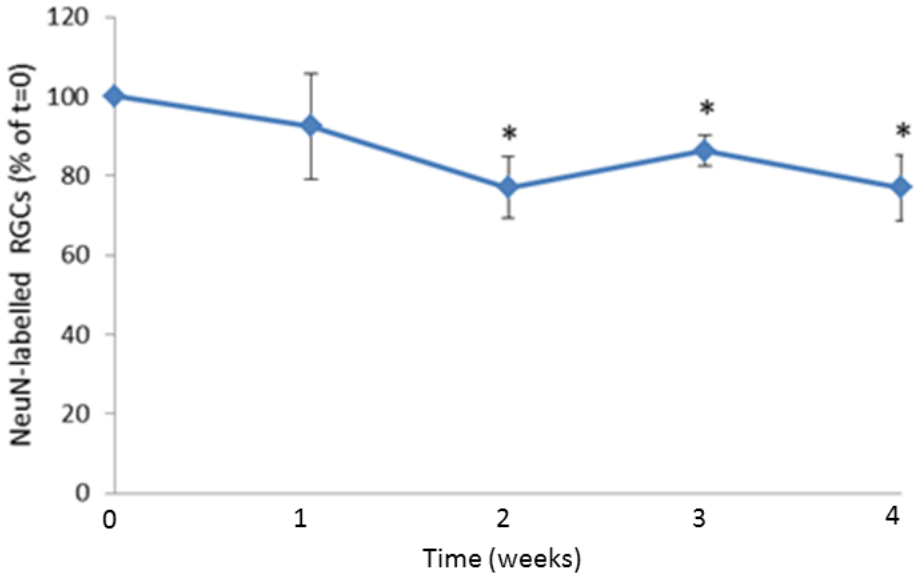


Figure 15: Timeline of NeuN-labelled RGC loss in HORCs with SF DMEM/HamF12 (mean±SEM, n=6). Results are presented as % of t=0. Student's t-test was used, *p<0.005.

Since the number of the RGCs showed minimal loss even after weeks 3 and 4 in culture, it was important to assess the viability of these remaining cells. The proportion of apoptotic RGCs was, therefore, estimated. There was a steep increase in the number of TUNEL-positive NeuN–labelled RGCs which reached a statistically significant level of approximately 75% at week 2 ($p<0.005$, $n=6$) and followed by a further gradual increase to approximately 80% by week 4 ($p<0.005$, $n=6$) (figure 16). The finding that a relatively stable count of NeuN-labelled RGCs was associated with a dramatic increase in the proportion of TUNEL-positive nuclei indicates that although the presence of cells is detectable by immunolabelling of cell somatas, the cells themselves are either dead or in the process of dying through apoptosis.

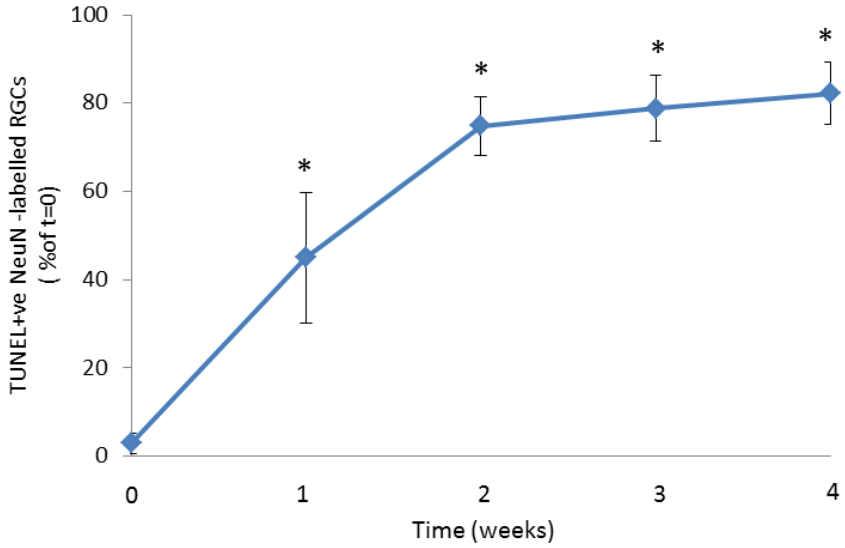


Figure 16: Proportion of TUNEL-positive NeuN-labelled RGCs in HORCs with SF DMEM/HamF12 (mean±SEM, n=6). Results are presented as % of t=0. Student's t-test was used, *p<0.005.

Previously, Niyadurupola and colleagues (2011) showed a decrease in the expression of *Thy1* mRNA of approximately 50% at 24 hours and a further 25% decrease at 48 hours, reaching a basal level after 72 hours in culture. THY1 is a cell surface glycoprotein that primarily associated with the RGC bodies, dendrites and axons (Osborne & Larsen 1996, Sheppard *et al.* 1991) and, thus, has been used extensively as a marker for RGCs. Before an assessment of *Thy1* mRNA expression, total RNA was extracted. Although the yield of total RNA decreased significantly at each time point compared with t=0 ($p<0.0005$, n=4) (figure 17), the amount of it was still sufficient to proceed with QRT-PCR. In HORCs cultured for 4 weeks, there was a statistically significant loss of *Thy1* mRNA expression that reached a basal level at week 1 ($p<0.0005$, n=4) (figure 18). The results were consistent with previously published findings from our group (Niyadurupola *et al.* 2011).

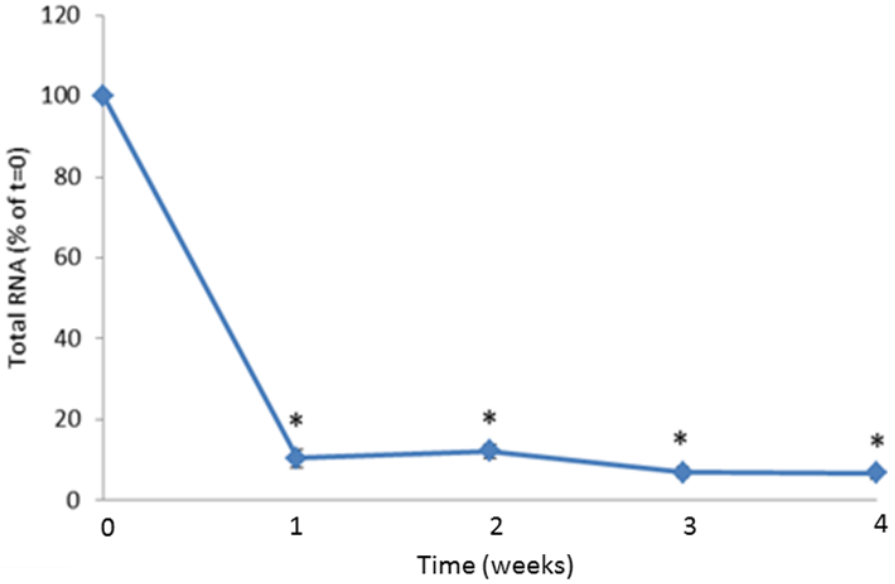


Figure 17: Total RNA loss in HORCs with SF DMEM/HamF12 (mean±SEM, n=4). Results are presented as % of t=0. Student's t-test was used, * $p<0.0005$.

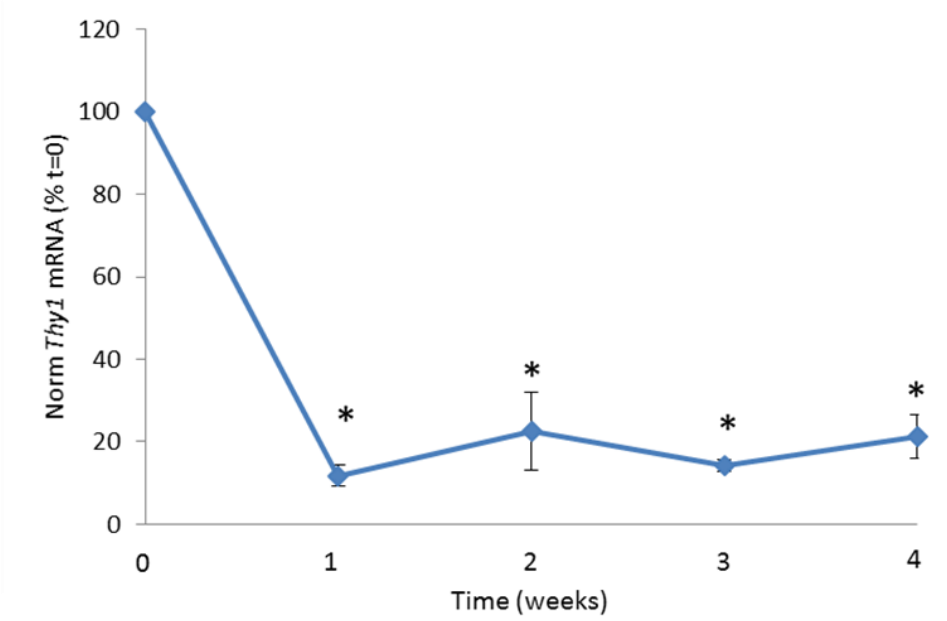


Figure 18: Loss of *Thy1* mRNA expression over time in HORCs with SF DMEM/HamF12 (mean±SEM, n=4). Results are presented as % of t=0. Student's t-test was used, * $p<0.0005$.

Optimisation of medium type in a long term HORC

Having established time dependant changes in long-term HORCs with SF DMEM/HamF12, the next step was to look at the effect of serum supplementation on RGC survival. In addition, since previous research indicated that Neurobasal medium with B27/N2 is the optimum condition for rat organotypic retinal cultures, the effect of this medium was also tested. To assess the cell layer organization and extent of overall cell death in HORCs in tested culture conditions, immunohistochemistry and TUNEL immunolabelling were performed (figure 19). At week 1, the laminar cell layer organization was preserved in HORCs under all culture conditions. Moreover, there were a few TUNEL-positive NeuN-labelled RGCs present in all culture conditions. However, there appear to be noticeably more of TUNEL-positive nuclei in the inner nuclear and outer nuclear layers in Neurobasal and 10% FCS DMEM/HamF12 media compared to that in SF DMEM/HamF12, with HORCs with Neurobasal medium having the highest level of TUNEL in all three retinal cell layers. Over week 2, 3 and 4, there was a gradual increase in the level of apoptosis in all three retinal cell layers under all culture conditions. However, HORCs in Neurobasal medium had the most intensive TUNEL staining starting from week 2, with a significantly higher number of apoptotic cells in all cell layers compared to that in DMEM/HamF12 with and without serum.

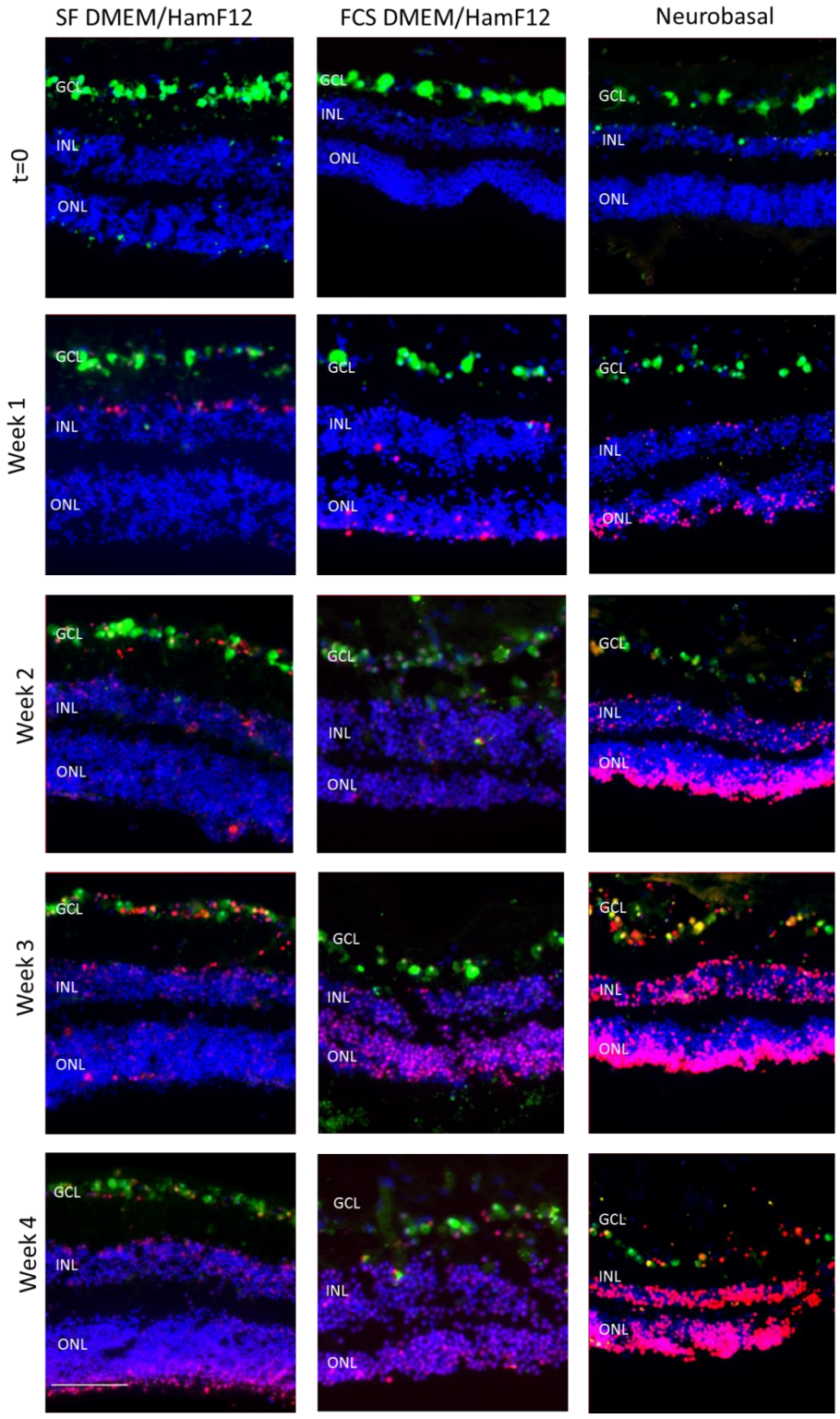


Figure 19: Representative images of immunohistochemistry and TUNEL-labelling of HORCs with DMEM/HamF12 in the presence or absence of 10%FCS, or with Neurobasal medium over 4 weeks in culture. NeuN (green) is a marker for RGCs, TUNEL (red) is a marker for apoptosis. Nuclei are stained with DAPI (blue). Scale bar, 100µm.

In order to establish the rate of the RGC loss in cultures with time, the number of NeuN-positive cells in the RGCL was counted at each time point (figure 20). Under all culture conditions, there was a significant decrease in the number of NeuN-labelled RGCs by weeks 2, 3 and 4 ($p<0.05$, $n\geq3$). The greatest RGC loss was observed in HORCs with Neurobasal medium, where a linear decrease reached a level of over 60% loss by week 3 ($p<0.0005$, $n=4$). Culture with DMEM/HamF12 medium, either with or without 10%FCS, resulted in a maximum loss of approximately 20% by weeks 2, and remained at the same level at week 3 and 4 ($p<0.05$, $n\geq3$). The difference between Neurobasal and DMEM/HamF12, with and without serum, reached statistical significance by weeks 3 and 4 ($p<0.05$, $n\geq3$).

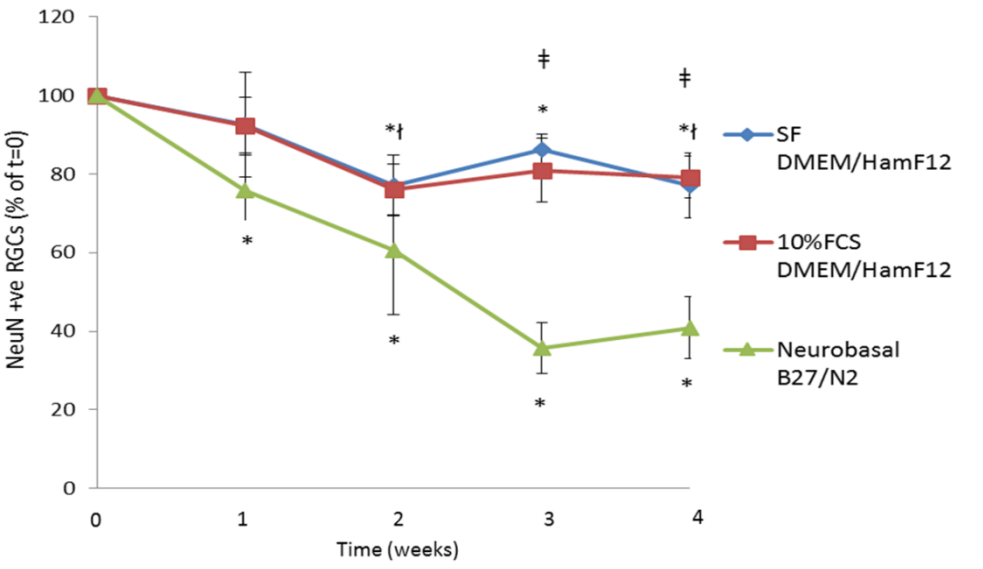


Figure 20: Loss of NeuN-labelled RGCs in HORCs with time. HORCs were cultured either in DMEM/HamF12, with or without serum, or Neurobasal medium supplemented with B27/N2 (mean±SEM, n≥3). All media contained gentamycin. Results expressed as % of t=0. In culture with Neurobasal medium, there was a linear loss of RGCs that reached a basal level at week 3, * $p<0.05$. In cultures with SF DMEM/HamF12, the loss of RGCs was gradual and reached statistical significance by week 2, * $p<0.05$. Cultures with 10%FCS DMEM/Ham F12 had the same pattern of RGC loss as cultures with DMEM/HamF12 without serum, ł $p<0.05$. The difference in a number of NeuN-labelled RGCs between cultures with DMEM/HamF12, either with or without serum, and Neurobasal media reached a statistically significant level at week 3 and 4, ǂ $p<0.05$. Analysis was made using one-way ANOVA with Dunnett's and Tukey's post-hoc tests.

TUNEL staining was used to identify the proportion of apoptotic NeuN-labelled RGCs. As can be seen in figure 21, under all conditions and at each time point, there was a significant increase in the number of TUNEL-positive NeuN–labelled RGCs ($p<0.05$, $n\geq3$). At week 1, the proportion of the apoptotic RGCs was smaller in HORCs with DMEM/HamF12 medium of about 50%, either with or without FCS, compared with that in HORCs with Neurobasal B27/N2 medium, where nearly 80% of RGC nuclei were labelled with TUNEL,

and the difference was statistically significant ($p<0.05$, $n\geq3$). However with time, the difference in levels of apoptosis of RGCs in HORCs under different culture conditions diminished. At week 4, the proportion of apoptotic RGCs reached around 90% in Neurobasal B27/N2 medium and FCS DMEM/HamF12, whereas the level of apoptosis of RGCs in SF DMEM/HamF12 has remained just under 80%.

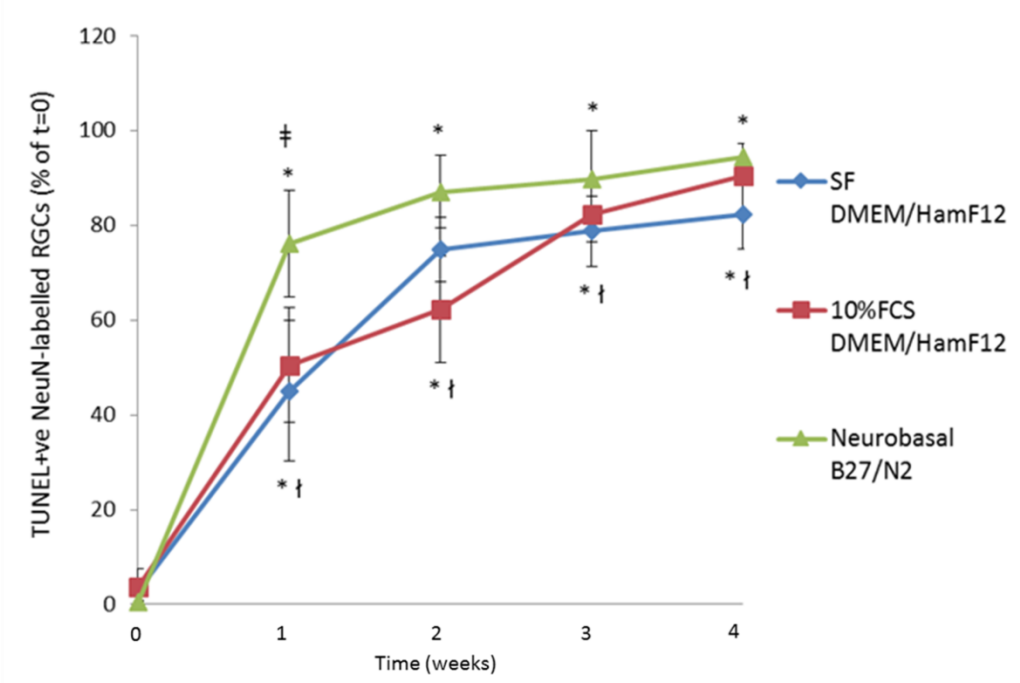


Figure 21: Proportion of TUNEL-positive NeuN-labelled RGCs in HORCs with DMEM/HamF12, either with or without serum, or Neurobasal medium supplemented with B27/N2 over 4 weeks in culture (mean±SEM, $n\geq3$). All media contained gentamycin. Results presented as % of t=0. All culture conditions caused a statistically significant increase in a number of RGC apoptosis each week, * ł $p<0.05$ vs t=0. Culturing with Neurobasal medium was associated with the steepest increase of nearly 80% at week 1, whereas HORCs with DMEM/HamF12 reached about 50% of RGC apoptosis at the same time point in culture, *$p<0.05$. The difference between Neurobasal and DMEM/HamF12, with and without serum, was statistically significant at week 1, ǂ $p<0.05$, and gradually diminished over weeks 2, 3 and 4. Analysis was performed using one-way ANOVA with Dunnett's and Tukey's post-hoc tests.

To assess the expression of RGC specific marker *Thy1*, the amount of total RNA was measured (figure 22). Under all culture conditions, the yield of total RNA reached a base line by week 2. Although the concentration of total RNA was low at week 2, 3 and 4, it was still sufficient to proceed with QRT-PCR in some samples allowing an estimation of *Thy1* mRNA expression at each time point under all culture conditions (figure 23). There was a significant decline in the level of *Thy1* mRNA expression at week 1 under all culture conditions ($p<0.05$, $n=4$). At week 2, 3 and 4, the level remained at approximately 20% of the post dissection level under all culture conditions. Despite a larger amount of total RNA at week 1 in HORCs cultured with Neurobasal B27/N2 medium, the expression of *Thy1* mRNA was similar to cultures with DMEM/HamF12, with or without serum. There was no difference in expression of *Thy1* mRNA in HORCs under all tested conditions.

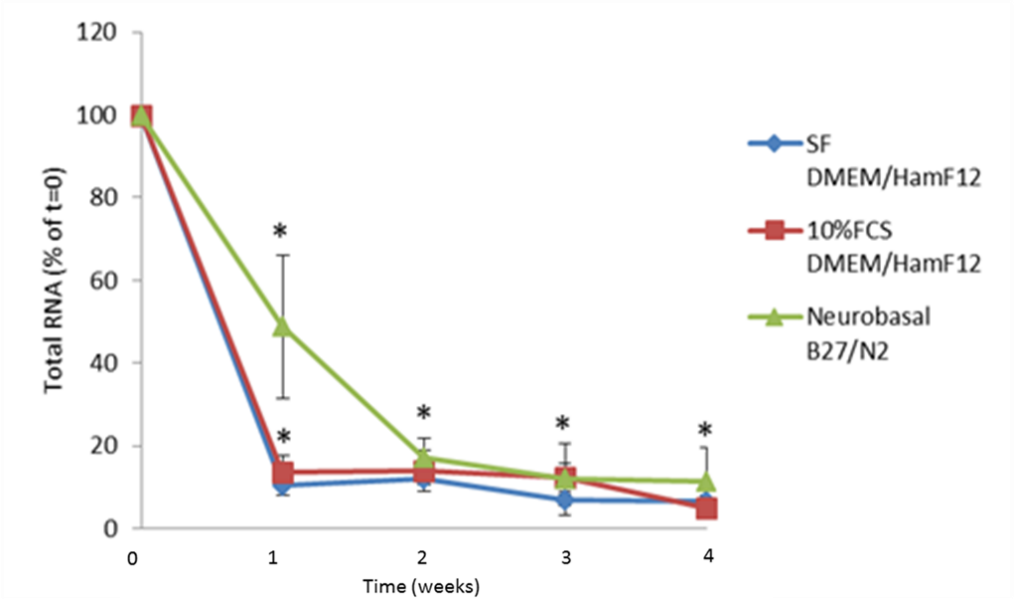


Figure 22: Loss of total RNA in HORCs over time. Results are presented as percentage of t=0 (mean±SEM, n=4). HORCs were cultured either in DMEM/HamF12, with or without serum, or Neurobasal medium supplemented with B27/N2. All media contained gentamycin. Results presented as % of t=0. Under all conditions and each time point, the loss of total RNA was statistically significant, * $p<0.05$. There was no statistically significant difference in amount of total RNA between different culture conditions. Analysis was performed using one-way ANOVA with Dunnett's and Tukey's post-hoc tests.

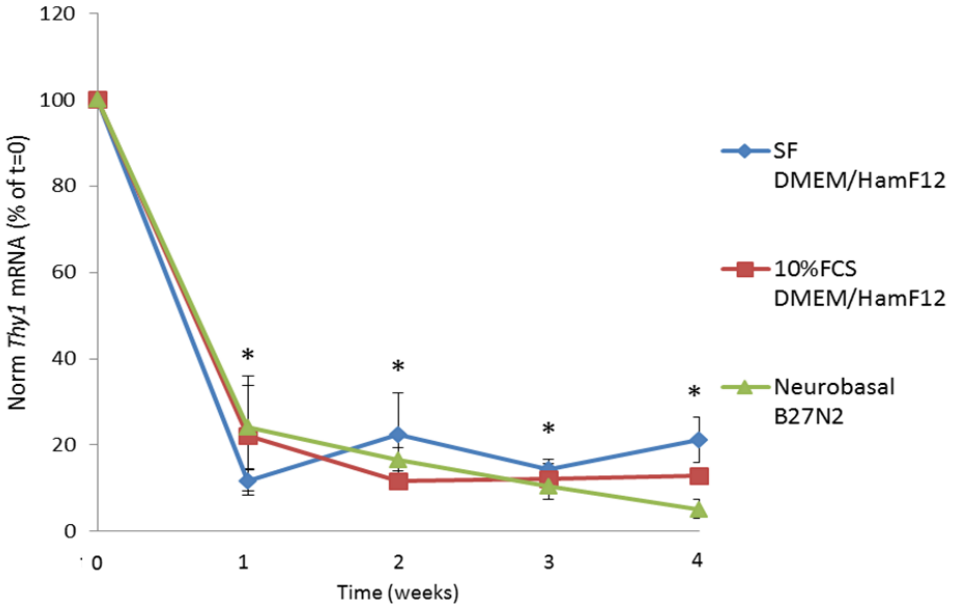


Figure 23: Loss of *Thy1* mRNA expression over time. Expression of *Thy1* mRNA was normalised to expression of *CYC1* and *TOP1* genes (mean±SEM, n=4). HORCs were cultured either in DMEM/HamF12, with or without serum, or Neurobasal medium supplemented with B27/N2. All media contained gentamycin. Results presented as % of t=0. Under all conditions, there was a statistically significant loss of *Thy1* mRNA expression, * $p<0.05$ vs t=0. There was no difference in expression of *Thy1* mRNA between different culture media. Analysis was performed using one-way ANOVA with Dunnett's and Tukey's post-hoc tests.

Optimisation of antibacterial agents in a long-term HORC

There is evidence that gentamycin possesses a toxic effect on the retina (Hancock et al. 2005), thus, it was important to compare the effect of different antibacterial agents on RGC survival in order to establish the optimum culture conditions for long-term HORCs. Two antibiotic supplements were tested: 50µg/ml gentamycin and a combination of 100U/ml penicillin with 100µg/ml streptomycin. Cultures with no added antimicrobial agents were also included. Levels of LDH released in culture media were assessed as a measure of cytotoxicity. After 24 hours, the lowest level of LDH appeared to be in cultures

with no added antibiotics and cultures with gentamycin had the highest level of LDH, although there was no statistical significance (figure 24).

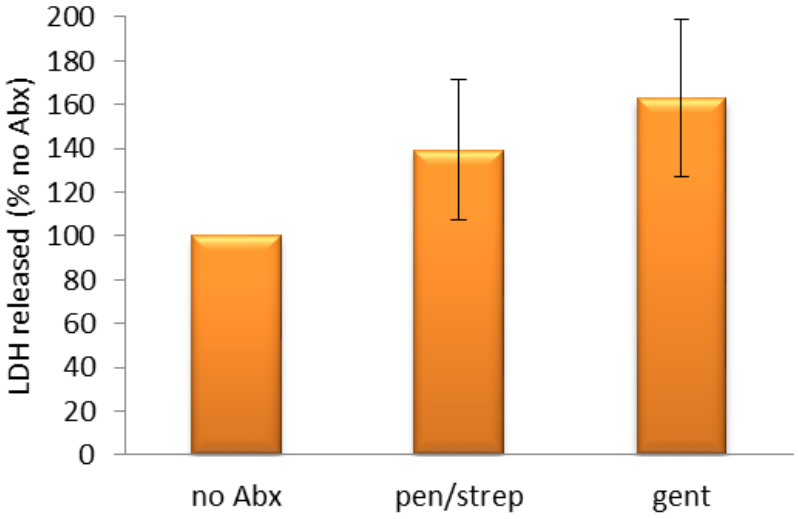


Figure 249: The relative level of cytotoxicity measured at 24 hours in HORCs cultured with SF DMEM/HamF12 and added either gentamycin (gent) or penicillin/streptomycin (pen/strep) or no antibiotics (no Abx) (mean±SEM, n=4). Student's t-test was used.

The expression of *Thy1* mRNA was also measured in HORCs under all tested conditions after 24 hours. As can be seen in figure 25, HORCs in SF DMEM/HamF12 with added gentamycin had the lowest amount of *Thy1* mRNA expression compared with cultures with added penicillin and streptomycin or no antibiotics and was statistically significant ($p<0.005$, n=4). HORCs with no added antimicrobial agent had the highest expression of *Thy1* mRNA. There was a statistically significant difference in *Thy1* mRNA expression between cultures with added gentamycin and no antibiotics ($p<0.05$, n=4).

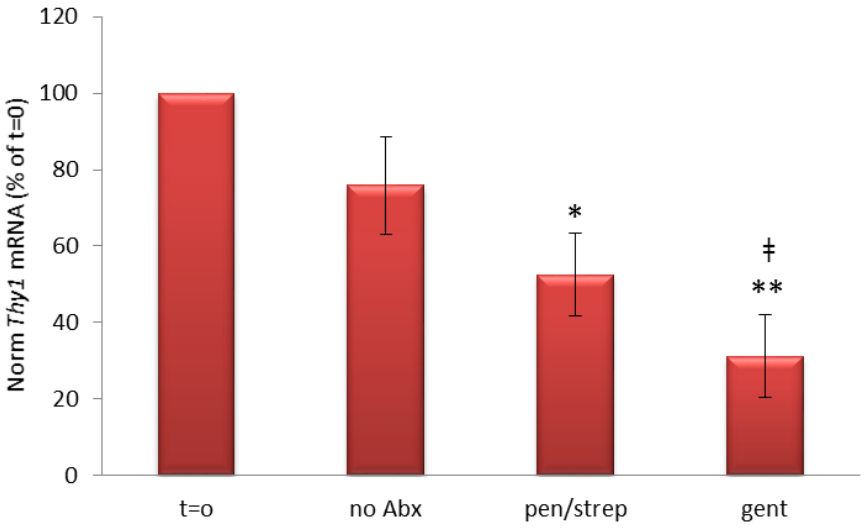


Figure 25: Normalised *Thy1* mRNA expression measured at 24 hours in HORCs cultured with SF DMEM/HamF12 with added either gentamycin (gent) or penicillin/streptomycin (pen/strep) or no antibiotics (no Abx) (mean±SEM, n=4). The results are normalized to expressions of housekeeping genes, *CYC1* and *TOP1*. There was a statistically significant *Thy1* mRNA loss in HORCs with added gentamycin compared with t=0, **p<0.005. In HORCs with penicillin/ streptomycin the loss of *Thy1* mRNA was less than in cultures with gentamycin, but still statistically significant compared with t=0, * p=0.001. The expression of *Thy1* mRNA with gentamycin was significantly lower compared to that with no antibiotics, ǂ p<0.05. Analysis was performed using one-way ANOVA with Dunnett's and Tukey's post-hoc tests.

Discussion

The present study aimed to determine whether HORCs could be maintained *ex vivo* in culture for longer than 96 hours, which was the longest culture period previously investigated by Niyadurupola and colleagues (2011). It also aimed to compare different culture conditions in order to prolong RGC survival in HORCs. In order to evaluate neurodegenerative changes in long-term HORCs, several RGC markers were used. In these experiments, the first indicator of the RGC fate was NeuN immunoreactivity. NeuN is a DNA-binding protein (Mullen *et al.* 1992) with isoforms present in neuronal cell nucleus and

cytoplasm (Lind *et al.* 2005, Wolf *et al.* 1996). Several studies have used NeuN to identify RGCs in human and animal retinal explants (Buckingham *et al.* 2008, Diaz *et al.* 2005, Niyadurupola *et al.* 2013, Niyadurupola *et al.* 2011). Niyadurupola and colleagues (2011) examined the number of NeuN-immunoreactive RGCs in short-term HORCs and documented no change in the count of these cells over the first 48 hours in culture, followed by a gradual decrease at the 72 and 96 hour time points. However, the authors demonstrated a reduction of approximately 40% in the NeuN-labelled RGC count after 60 min of OGD followed by 23 hours of reperfusion compared to that in controls (Niyadurupola *et al.* 2011). In long-term HORCs, NeuN-immunolabelling of cells in the GCL demonstrated the presence of RGCs under all culture conditions throughout the 4 week culture period. There was no statistically significant change in the number of NeuN-labelled RGCs until the week 2 with DMEM/HamF12, either with or without serum, when it reached a basal level of about 20% loss. Interestingly, the loss of RGCs in HORCs with Neurobasal medium supplemented with B27/N2 was greater than in DMEM/HamF12, either with or without serum, at each time point in culture. The results of this chapter also showed that here was no correlation between the presence of NeuN-labelled RGCs on immunohistochemistry and the expression of *Thy1* mRNA. *Thy1* is a cell surface glycoprotein that primarily associated with the RGC bodies, dendrites and axons (Osborne & Larsen 1996, Sheppard *et al.* 1991) and, thus, has been used extensively as a maker for RGCs (Schlamp *et al.* 2001). In long-term HORCs, a dramatic loss of *Thy1* mRNA expression was documented at week 1, as was expected based on results by Niyadurupola and

colleagues (2011), indicating that the human retinal explants can be cultures for up to one week if the experiment is based on the measure of the retinal cell gene markers alone. In current study, the other markers for evaluation of the retinal cell fate were also used. Quantitative analysis of TUNEL-positive NeuN-labelled RGCs revealed a steep increase in the number of dying RGCs by apoptosis under all culture conditions of approximately 80% after 2 weeks in culture, with the steepest increase being found in HORCs with Neurobasal medium, suggesting this time period to be the most sensitive for evaluating the change in the NeuN-labelled TUNEL-positive RGC count. The data presented therefore also suggests that not only the use of several markers is important in order to identify the fate of RGCs, but also that the most sensitive marker of the RGC survival in a short-term HORC might be the expression of *Thy1* mRNA. In a long-term HORC, however, co-labelling of RGCs with TUNEL and NeuN would be the most reliable way of quantitative analysis of the RGC survival. Our findings are supported by the data from another group demonstrating a delay in NeuN-labelled RGC loss when compared to RGC-specific gene marker expression and highlighting the importance of using several markers in assessment of the RGC fate. Buckingham and colleagues (2008) comprehensively demonstrated that NeuN is a reliable marker for RGCs by co-labelling mouse retinal tissue *in vitro* with NeuN and retro-loaded fluorogold, as RGC markers, together with ChAT and GAD-67, as markers for amacrine cells. The authors also showed that the neuronal somata continuously exerted NeuN-immunoreactivity throughout glaucomatous RGC degeneration despite progressive loss of RGC axons in the ONH and a significant down

regulation of the RGC-specific mRNA expression using a congenital mouse model of glaucoma (Buckingham *et al.* 2008), indicating a delay in the NeuN-labelled RGC disappearance. The results, however, were limited by the lack of TUNEL data.

Dulbecco's modified Eagle's medium (DMEM) is a modified Eagle's minimum essential medium (EMEM) that has almost twice the concentration of amino acids and four times the amount of vitamins, as well as ferric nitrate, sodium pyruvate, and additional amino acids. DMEM is a basal medium and contains no proteins or growth promoting agents and, therefore, is used in a 1:1 combination with defined Ham's nutrients mixture (HamF-12) (Ham 1965, Jayme et al. 1997). Neurobasal medium has been developed as a modified DMEM/HamF12 medium specifically for cultures of isolated rat embryonic hippocampal neurons, in which the osmolality and the concentration of several amino acids were reduced and several compounds were eliminated, such as ferrous sulphate and glutamate (Brewer et al. 1993). The authors have also demonstrated that Neurobasal medium supplemented with B27 suppressed the growth of glial cells in the neuronal cell culture (Brewer et al. 1993), indicating the potential limitation of this medium for the use in tissue cultures. Since then, some researchers, when using Neurobasal medium, explored the ability of this medium to suppress glial cells in their experiments or reported unexplained results. Bonde and colleagues have noticed unexplained aggravation of neuronal cell death associated with pre- treatment of rat hippocampal slice cultures with glial cell derived growth factor (GDGF) prior to OGD experiments with Neurobasal medium (Bonde et al. 2003). Montero and

colleagues(2009) have discovered that switching to Neurobasal medium supplemented with B27 before experiments of OGD induced a significant cell death in mouse hippocampal slice cultures (Montero et al. 2009). The B27 and N2 supplements are chemically defined and commercially produced nutrient mixtures designed as modifications of older versions of nutrient mixtures to enhance specifically the survival of neuronal cells in culture (Brewer et al. 1993, Wang et al. 2002). Neurobasal medium supplemented with B27/N2 is a common condition for culturing rat and mouse retinal explants (Johnson & Martin 2008, Manabe et al. 2002, Wang et al. 2002). However, there are concerns raised by some researchers questioning the content of B27 supplement, since poor neuronal cell survival and maturation was noticed in cultures with this supplement (Cressey 2009). To support the survival and proliferation of cultured cells, supplementation of a basal medium with serum is widely used. However, in long-term HORCs, there was no difference in the number of viable RGCs at any of the measured time points between cultures with serum- free and serum supplemented DMEM/HamF12.

FCS is complex mixture of different factors, like proteins, growth factors, vitamins and hormones, which are essential for the cells growth and maintenance in culture. However, the complex undefined nature of serum is a potential problem when assessing the effect of regulatory agents, such as hormones or neurotransmitters. The inclusion of serum may significantly affect experimental reproducibility due to differences in serum composition produced by batch-to-batch variations. Moreover, there are concerns regarding not only inhumane ways of serum collection from unborn calves (Van der Valk *et al.*

2004), but also regarding the risk of contaminations, for example with viruses, when animal products are used in culture (Wessman & Levings 1999). In fact, the use of animal substrates is strongly discouraged in biological medical productions (Schiff 2005, Van der Valk *et al.* 2004). Attempts have been made to develop standards for *in vitro* cell and tissue cultures by introducing guidelines for good cell culture practice (GCCP) recommending the use of serum-free media, however it is not legally binding (Van der Valk *et al.* 2010). It follows therefore from the above observations that SF DMEM/HamF12 medium is the most appropriate choice for long-term HORCs, the medium used by Niyadurupola and colleagues (2011, 2013) for short-term HORCs.

It is generally accepted that there is a need for the use of antibacterial agents in cultures in order to prevent bacterial contamination of the medium. Penicillin/streptomycin combination and gentamycin are commonly used antibiotics in culture. Gentamycin is an aminoglycoside that is effective against gram-negative bacteria by inhibiting bacterial protein synthesis (Vakulenko & Mobashery 2003). Use of gentamycin in medicine has been limited due to drug ototoxicity (Jackson & Arcieri 1971) and nephrotoxicity (Wilfert *et al.* 1971). From clinical studies, minimum inhibitory concentration of gentamycin is known to be at the level of 2µg/ml (Noone *et al.* 1974). The authors have also advised on a reduction of the dose if the serum concentration of gentamycin exceeded 15µg/ml in order to avoid toxic effects to ears and kidneys in patients with sepsis (Noone *et al.* 1974). In ophthalmology, gentamycin used to be one of the most commonly used antibiotics for intravitreal injections to treat endophthalmitis (Penha *et al.* 2010). However, there are several reports

regarding gentamycin toxicity in the eye. The toxic effect of gentamycin on viability of the corneal endothelium has been demonstrated in an *in vivo* rabbit model, where intracameral injection of gentamycin at concentrations between 20µg/ml and 20mg/ml has caused a dose dependant loss of endothelial cells between 22% and 67%, respectively (Kobayakawa *et al.* 2010). In rabbit and rat retinal cultures, gentamycin has been found to affect electrophysiological responses due to toxic effect on bipolar and horizontal cells, as well as diffusely disrupted the NFL and IPL (Hancock *et al.* 2005). In addition, there are several case reports of patients who lost their vision due to retinal ischaemia following topical and intravitreal administrations of gentamycin (Campochiaro & Conway 1991, Kanter & Brucker 1995). Because of potential gentamycin toxicity to the retinal tissue, the effect of different antibiotics, specifically penicillin/streptomycin in combination or gentamycin, on the RGC survival in HORCs was assessed and compared with HORCs without antibiotics. The concentration of gentamycin routinely used in HORCs was 50µg/ml which is above the minimum inhibitory dose and within the range of the safe dosage to avoid a potential toxic effect to the retina. A combination of 100U/ml penicillin and 100µg/ml streptomycin was the same as reported by other groups in routine cell and tissue cultures (Carter & Dick 2003, Johnson & Martin 2008, Van Bergen *et al.* 2009). In HORCs with gentamycin, not only was the loss of *Thy1* mRNA expression statistically significant in comparison with cultures without antibiotics, but also the level of total cell death was higher, but not significantly, when LDH activity was measured in a 24 hour culture. Although cultures with penicillin/streptomycin had some reduction in

Thy1 mRNA expression, it was still not as significant as in HORCs with gentamycin when compared with antibiotic-free cultures. LDH activity was also lower, but not significantly, in medium of HORCs with penicillin/streptomycin compared with added gentamycin cultures. In literature, when both types of antibiotics were compared in bone marrow-derived mesenchymal stem cell (bmMSC) cultures in medium with added 100mg/ml of gentamycin or 1% mixture of penicillin and streptomycin or hyaluronic acid, it has been shown that there was a loss of more than 95% of cell viability in culture with gentamycin, whereas in culture with penicillin/streptomycin as well as hyaluronic acid, the viability of the cells was preserved in more than 80% of cells (Bohannon *et al.* 2013). The data presented therefore suggests that not only penicillin and streptomycin are the least toxic antibiotics in culture, but also that it is important to avoid the use of gentamycin in experiments involving bmMSCs, since there is an increasing interest in neuroprotective potential of bmMSC (Johnson *et al.* 2010). Interestingly, it was found that HORCs without antibiotics had the smallest loss of *Thy1* mRNA expression after 24 hours in culture, as well as the lowest, but not significantly, level of total cell death. Although there are other studies that cultured human retinal explants for up to 14 days in medium with no added antibiotics (Knott *et al.* 1999, Mayer *et al.* 2005), the use of antimicrobial agent with the least toxic effect on the retinal tissue would be feasible, especially in long-term HORCs when a complete isolation of each individual experiment is not practical and the risk of contamination would always be present and can be devastating due to a limited number of human retinal explants.

To summarize, the aims of the presented work were to characterise a long-term model of the RGC degeneration using HORCs and then to develop an optimum culture condition in order to establish this model for future used in neuroptotective studies, specifically in glaucoma. It was found that SF DMEM/HamF12 is the condition of choice for a long-term HORC and all subsequent experiments were carried out using this culture medium. Moreover, it was demonstrated that a combination of penicillin and streptomycin is a better choice of antibiotics for HORCs compared with gentamycin, however, an antibiotic free culture would be a feasible option as well.

CHAPTER 4

A ROLE OF MESENCHYMAL STEM CELL DERIVED NEUROTROPHIC FACTORS IN PROLONGING RGC SURVIVAL IN HUMAN ORGANOTYPIC RETINAL CULTURES

Introduction

Previous experiments, presented in chapter 3, established that SF DMEM/HamF12 with added penicillin and streptomycin is the medium of choice for long-term culture of human retinal explants. The next aim was to identify specific neurotrophic factors that might be able to prolong the survival of RGCs in long-term culture. Currently, there is a great degree of interest in the trophic and protective qualities that bone marrow derived mesenchymal stem cells (bmMSCs) exert on cells and tissues throughout the body, including RGCs (Johnson *et al.* 2010, Yu *et al.* 2006a). In the retina, production of neurotropic factors after MSC transplantation has been implemented as a main mechanism by which these cells confer neuroprotection (Li *et al.* 2009, Yu *et al.* 2008, Zhao *et al.* 2011, Zwart *et al.* 2009).

It has been long established that MSCs are a source of an array of growth factors of various concentrations with multiple mechanisms of action, including neuroprotection (Hsieh *et al.* 2013, Johnson *et al.* 2014), angiogenesis (Estrada *et al.* 2009, Hsieh *et al.* 2013), and immunomodulation (Parekkadan *et al.* 2007). However, characterisation of the MSC secretome is still ongoing (Kupcova-Skalnikova 2013) and new, previously unknown factors are continuously emerging (Estrada *et al.* 2009, Johnson *et al.* 2014).

Based on information available at the time of the conduction of experiments (K. Martin, personal communication; (Johnson *et al.* 2014)), the decision was made to investigate bmMSC-derived neurotrophic factors because of their potential to prolong RGC survival on the human model of the RGC degeneration, such as leukaemia inhibitory factor (LIF) (Nasef *et al.* 2008), platelet derived growth factor (PDGF) (Ball *et al.* 2012) and vascular endothelial growth factor (VEGF) (Beckermann *et al.* 2008, Kinnaird *et al.* 2004).

Leukaemia inhibitory factor (LIF) is a highly glycosylated 40–50 kDa glycoprotein from the interleukin 6 (IL-6) cytokine family (Heinrich *et al.* 2003) and is a marker of MSC differentiation potential (Whitney *et al.* 2009). LIF receptor consists of two subunits: gp130 is a common subunit for all IL-6 family of cytokines, and another specific for LIF receptor (LIFR) subunit (Heinrich *et al.* 2003). It has been reported that LIF signal transduction is facilitated via the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway (Heinrich *et al.* 2003), as well as mitogen-activated protein kinase (MAPK) (Heinrich *et al.* 2003, Park *et al.* 2003) and phosphatidylinositol 3-kinase (PI3K)/Akt signalling cascades (Alonzi *et al.* 2001). LIF is known to be expressed and secreted by multiple tissues and cell types, and has a wide array of actions. For example, LIF acts as a stimulus for proliferation of hematopoietic cells (Leary *et al.* 1990), and plays a crucial role in embryogenesis (Ware *et al.* 1995) and fertility (Chen *et al.* 2000). In murine embryonic stem cell culture, it has been shown that LIF is a crucial factor in promoting cells proliferation (Bauer & Patterson 2006, Williams *et al.* 1988),

and deprivation of the factor causes cell apoptosis and differentiation of the surviving cells (Duval *et al.* 2000). It has also been shown that LIF promotes glial cell proliferation and astrocytic differentiation (Bauer & Patterson 2006, Mi *et al.* 2001, Nakanishi *et al.* 2007). Numerous reports using *in vivo* animal models (Blesch *et al.* 1999, Chollangi *et al.* 2009, Leibinger *et al.* 2009, Suzuki *et al.* 2005), as well as *in vitro* studies using cell cultures (Han *et al.* 2013, Leibinger *et al.* 2009, Majumder *et al.* 2012, Martinou *et al.* 1992, Yamamori *et al.* 1989) have demonstrated the potential neurotrophic and neuroprotective effects of LIF. However, an exact origin for the regenerative potential of LIF has not been elucidated. Suggested mechanisms involve the recruitment of neural stem cells (NSCs) (Bauer & Patterson 2006), stimulation of neurotrophic factors production (Blesch *et al.* 1999), and direct action on neurons (Leibinger *et al.* 2009). Neurotrophic and neuroprotective effects of LIF have been demonstrated in the brain (Suzuki *et al.* 2005), spinal cord (Blesch *et al.* 1999), and the retina (Chollangi *et al.* 2009). Neurotrophic property of LIF has been shown using an *in vivo* rat model following spinal cord injury, where grafting of genetically modified fibroblast designed to produce high amount of LIF enhanced the expression of neurotrophin-3 with a significant increase in axon outgrowth (Blesch *et al.* 1999). However, the effect on nerve function has not been investigated in this study. Another study, using an ischaemic brain injury model, has shown that both neurological deficit and ischaemic damage were much less severe after exogenous LIF injection in a dose dependant manner (Suzuki *et al.* 2005). It has also been demonstrated *in vitro* that LIF promotes survival of motoneurons (Martinou *et al.* 1992),

cutaneous sensory neurones (Horton *et al.* 1998), and autonomic neurons of the heart (Yamamori *et al.* 1989). The potential involvement of LIF in the retinal pathophysiological processes has also been investigated. Stimulation of inflammatory pathways by means of optic nerve crush and intraocular lens injury has been shown to induce a robust upregulation of LIF by mouse retinal astrocytes (Leibinger *et al.* 2009). The authors have also demonstrated that exogenous LIF can stimulate axon regeneration in the optic nerve *in vivo* and induce RGC outgrowth *in vitro* (Leibinger *et al.* 2009). A role of LIF in glaucoma has been investigated using an *in vivo* rat model, where an increase in *LIF* expression of 223% was observed in the optic nerve head compared with controls after 5 weeks of acute IOP elevation (Johnson *et al.* 2011). Another *in vivo* study has demonstrated that light preconditioning caused a strong upregulation of LIF expression in the mouse retina, which was protective to photoreceptors (Chollangi *et al.* 2009). It follows from the above observations that LIF possesses a neurotrophic effect, however, there is rather limited evidence for this effect to RGCs.

Platelet derived growth factor (PDGF) is another important growth factor produced in abundance by MSCs, that plays a key role in MSC proliferation and differentiation (Ball *et al.* 2012). The PDGFs are a family of proteins consisting of PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD (Kazlauskas 2000, Li & Eriksson 2003) that are members of a PDGF/VEGF superfamily. The structure of PDGF protein is dimeric, and both PDGF-CC and PDGF-DD share the structure of their second subunit with vascular endothelial growth factor (VEGF) (Reigstad *et al.* 2005). PDGF-CC and DD

have to be proteolytically activated with tissue plasminogen activator (tPA) and/or plasmin to allow receptor binding, whereas other PDGFs do not require proteolytic activation (Fredriksson *et al.* 2005, Lei & Kazlauskas 2008). Secreted PDGFs signal through tyrosine kinase receptors, that are made of homo- and heterodimers of PDGF receptor α and β (Reigstad *et al.* 2005). PDGF-BB is the universal ligand, whereas PDGF-AB and PDGF-CC activate both PDGFR-αα and PDGFR-αβ, and PDGF-DD activates both PDGFR-β and PDGFR-αβ. PDGF-AA is the most selective member of the PDGF family and exclusively activates PDGFR-αα (Lei *et al.* 2010). Several genetically modified mouse models have been developed where *PDGF-A* and PDGF-*B*, as well as *PDGF* receptor -*α* and -*β* genes were knock-out to demonstrate a crucial role of these factors not only in embryonic development, but also their involvement in physiological and pathological processes in mesenchymal tissues (Fruttiger *et al.* 1999, Ishii *et al.* 2006, Leveen *et al.* 1994, Soriano 1997). For example, in a study using *PDGF-B* knock-out mice, perinatal death was associated with severe kidney and vascular smooth muscle abnormalities (Leveen *et al.* 1994). Although some pups of *PDGF-A* mutant mice have survived after birth, a severe loss of myelin in spinal cord, optic nerve and cerebellum regions has indicated the importance of *PDGF-A* for oligodendrocyte development (Fruttiger *et al.* 1999). Perinatal death has also been observed in *PDGF-α* receptor gene knock-out mice and was associated with incomplete cephalic closure (Soriano 1997). The neuroprotective effect of PDGFs has been demonstrated by several *in vivo* studies suggesting that these factors regulate the susceptibility of CNS neurons to cell death after injury

(Egawa-Tsuzuki *et al.* 2004, Ishii *et al.* 2006). Silencing of *PDGF-β* receptor gene in mice has accelerated the loss of brain neurons after NMDA-excitotoxic and cryogenic injuries (Ishii *et al.* 2006). Pre-treatment with PDGF-BB has been demonstrated to confer neuroprotection against NMDA mediated excitotoxic insult in the brain of rats, whereas inhibition of PDGF-BB secretion has exacerbated neuronal cell death (Egawa-Tsuzuki *et al.* 2004). The ability of PDGF-AA to attenuate oxidative stress-induced cell death via PI3K/Akt pathway has also been demonstrated in the RGC-5 cell culture (Kanamoto *et al.* 2011). The results of the above studies strongly indicate that PDGFs have a potential to extent RGC survival in the retina.

Vascular endothelial growth factor (VEGF) is a major regulator of blood and lymphatic vessel development (Breier *et al.* 1992, Karkkainen *et al.* 2004). The main functions of VEGFs are in regulation of endothelial cells proliferation, migration, and vascular tube formation (Kinnaird *et al.* 2004). In mammals, the VEGF family consists of five dimeric glycoprotein growth factors: VEGF (or VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) (Tammela *et al.* 2005). VEGF-A is the most abundant and most well studied VEGF. By differential mRNA splicing, the single *VEGF-A* gene is represented by three major isoforms throughout most tissues in humans: VEGF121, VEGF165 and VEGF189. whereas in the mouse, the isoforms are VEGF120, VEGF164 and VEGF188 (Ruhrberg 2003). The structure of these VEGF forms differ primarily in the number of amino acids and presence or absence of the heparin binding domains, giving rise to forms that differ in their heparin and heparan-sulfate binding ability. All VEGF-A isoforms bind with high affinity

to two transmembrane tyrosine kinase receptors VEGFR1 (fms-like tyrosine kinase 1; FLT1) and VEGFR2 (also known as foetal liver kinase 1 (FLK1)/ kinase domain region (KDR)). The receptors signal predominantly by activating the MAPK/ERK (extracellular signal-regulated kinase) pathway (Ferrara 2001, Ma *et al.* 2011). All VEGF isoforms bind to the Flt-1 and Flk-1 receptor tyrosine kinases (Ruhrberg 2003). There are also two other non-tyrosine kinase co-receptors from the neuropilin family, Nrp-1 and Nrp-2, that are selective for certain isoforms (Gluzman-Poltorak *et al.* 2000). For example, the heparin-binding domain of VEGF165 facilitates binding to Nrp-1, whereas VEGF121 cannot bind to either receptors due to the lack of any heparin-binding domain (Gitay-Goren *et al.* 1996, Gluzman-Poltorak *et al.* 2000). There are multiple reports demonstrating neurotrophic effect of bmMSCs *in vivo* via significant upregulation of VEGF secretion in the ischaemic brain injury in rats that promoted endogenous neurogenesis, reduced apoptosis and improved functional recovery (Bao *et al.* 2011, He *et al.* 2011, Li *et al.* 2014, Wakabayashi *et al.* 2010, Wei *et al.* 2012). Several studies have also demonstrated the neurotrophic properties of VEGF *in vitro*. It has been found to rescue neurons after serum withdrawal in culture (Jin *et al.* 2000a), and promote neuronal survival in both the foetal organotypic cortical explants (Rosenstein *et al.* 2003) and cultured adult mouse peripheral nervous system ganglia neurons (Sondell *et al.* 1999). Moreover, the ability of exogenous VEGF to rescue neuronal cells has been demonstrated in hypoxia (Jin *et al.* 2000b, Svensson *et al.* 2002), mechanical injury (Ma *et al.* 2011) and glutamate excitotoxicity (Bogaert *et al.* 2010, Svensson *et al.* 2002). Due to

lack of vessels in neuronal cell and tissue cultures of *in vitro* experiments, it is possible to conclude that the neuroprotective effect of VEGF can potentially be, at least in part, vessel independent.

Considering that the results of several studies have extensively demonstrated the neurotrophic effect of the bmMSC derived growth factors LIF, PDGF and VEGF, the aim of the experiments presented in this chapter was to determine whether they could prolong RGC survival in the long-term culture of the human retina.

Results

LIF has no effect on RGC survival in a long- term HORC

In order to analyse the possible beneficial effect of LIF supplementation on RGC survival, the effect of LIF at concentrations of 10, 30 and 50ng/ml was investigated in a long-term HORC. As can be seen in figure 26, there was a well-defined preservation of the retinal cell layer architecture under all culture conditions. The TUNEL-positive cells were present not only in the GCLs but also in the other nuclear layers in all cultures. However, in HORCs with 30 and 50ng/ml, it appeared to have more apoptotic cells in the ONL. As expected from previous experiments (chapter 3), quantitative analysis of NeuN-immunoreactive RGCs (figure 27) showed no difference in RGC counts, indicating that LIF did not cause a loss of RGCs. Therefore, the labelling with TUNEL was also carried out and the proportion of NeuN-labelled TUNEL-positive RGCs was quantified. There was a noticeable increase in the level of

apoptosic RGCs in cultures with 30 and 50ng/ml LIF compared to that of controls, however the difference was not significant (figure 28).

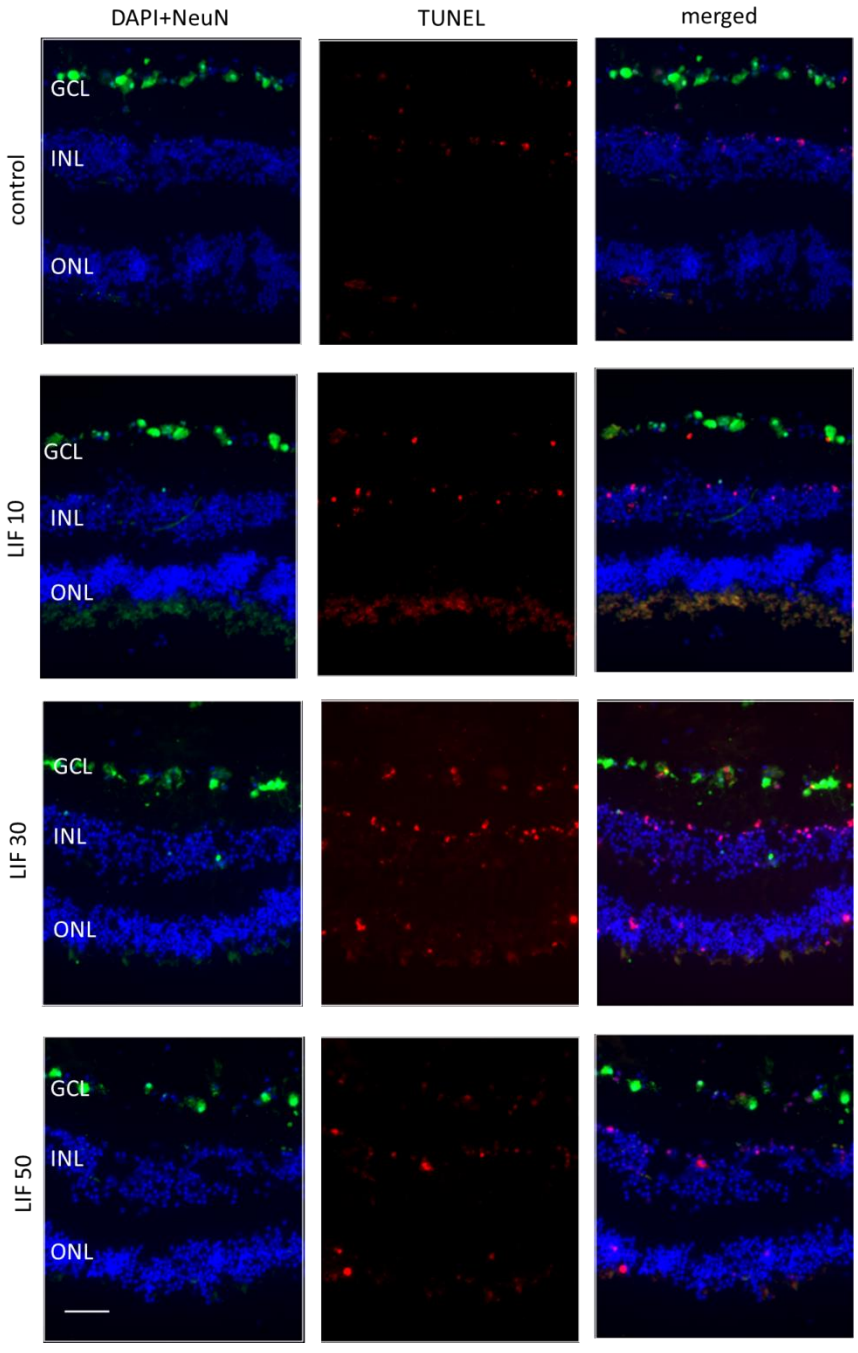


Figure 26: Representative images of HORCs after 1 week culture with LIF at concentrations of 10, 30 and 50ng/ml. RGCs are labelled with NeuN (green), apoptotic cells with TUNEL (red), nuclei with DAPI (blue). Scale bar, 100µm.

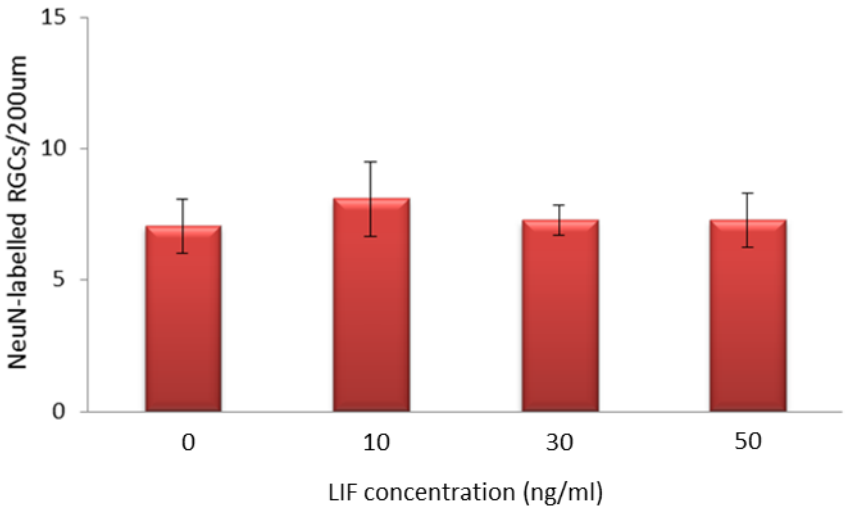


Figure 27: NeuN-labelled RGC count after 1 week in culture with LIF at concentrations of 10, 30 and 50ng/ml (mean±SEM, n=4). Student's t-test was used.

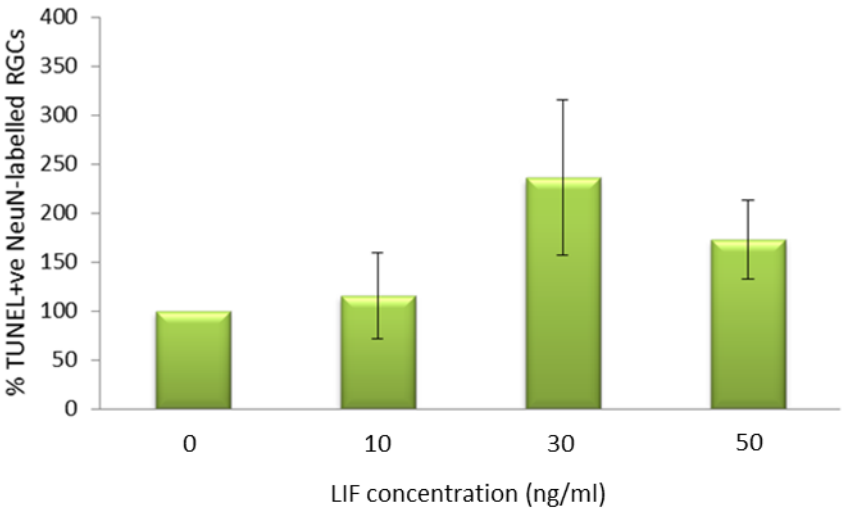


Figure 28: Proportions of NeuN-labelled TUNEL-positive RGCs after 1 week in culture with LIF at concentrations of 10, 30 and 50ng/ml (mean±SEM, n=4). Student's t-test was used.

PDGF-AA and VEGF prolong RGC survival in a long-term HORC

To determine a potential neurotrophic effect of PDGF-AA, PDGF-AB and VEGF on RGC survival in long-term HORCs, all growth factors were tested at concentration of 50ng/ml. At 1 week, as can be seen in figure 29, the structure of retinal explant cell layers was well preserved in controls and treated retinal explants. To assess the overall cell death level in HORCs at week 1, LDH activity was measured and appeared to be lower in all explants treated with growth factors compared to that in controls (figure 30). In fact, LDH activity in media of HORCs treated with VEGF was significantly lower compared to that in controls ($p<0.05$, $n=6$). Although the reduction in the level of overall cell death in cultures with PDGF-AA and PDGF-AB was also noticed, it was not statistically significant. Quantification of NeuN-immunoreactive RGCs was carried out and showed no difference in the number of RGCs in treated HORCs compared to that in controls (figure 31). TUNEL was also carried out and quantified. As can be seen in figure 32, the proportion of NeuN-labelled TUNEL positive RGCs was also lower in HORCs treated with PDGF-AA, -AB and VEGF compared to that in controls reaching statistical significance for PDGF-AA and VEGF($p<0.05$, $n=6$). The reduction in the proportion of apoptotic RGCs was over 30% in HORCs cultures with VEGF and over 20% in cultures with added PDGF-AA.

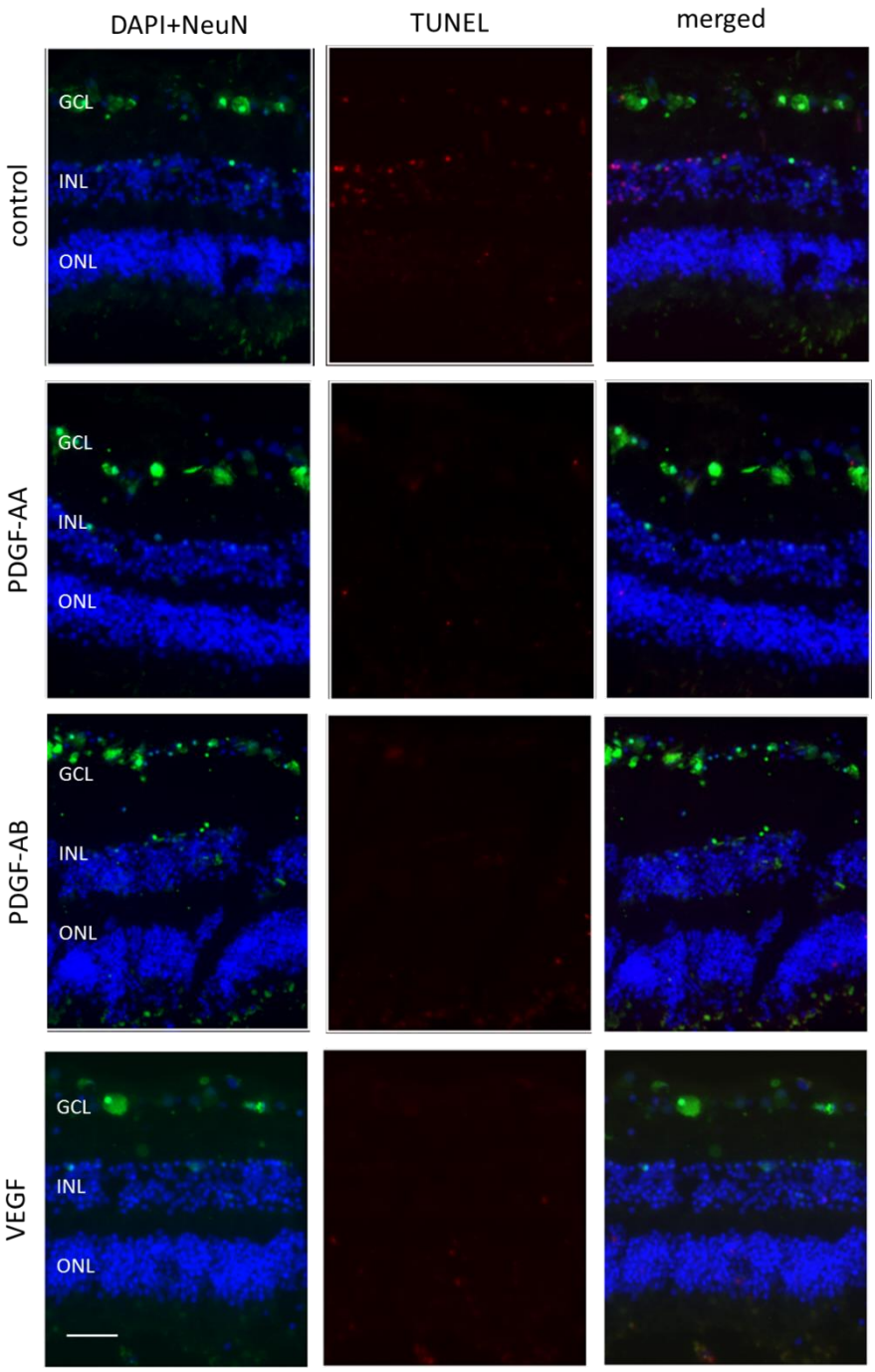


Figure 2910: Representative images of HORCs after 1 week treatment with PDGF-AA, PDGF-AB and VEGF at concentration of 50ng/ml and vehicle control. RGCs were labelled with NeuN (green), cells nuclei were labelled with DAPI (blue), TUNEL-positive nuclei are red. Scale bar, 100µm.

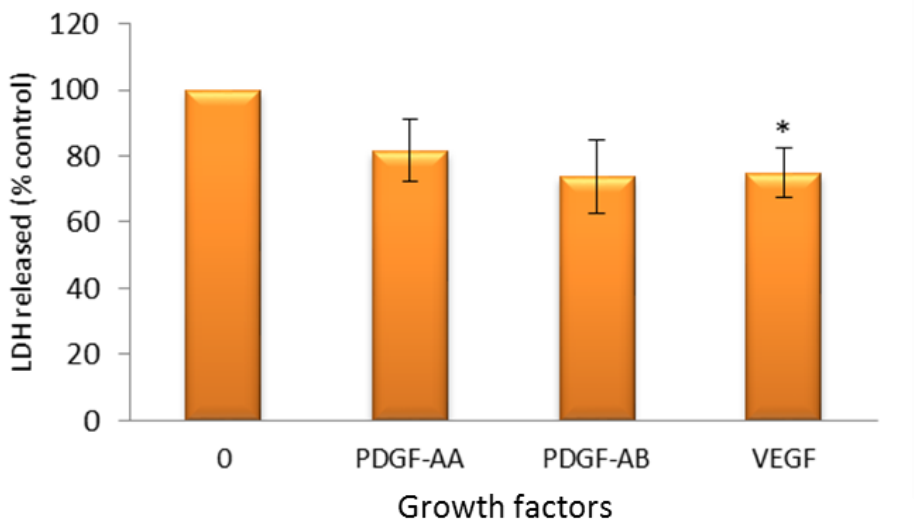


Figure 30: The relative level of LDH released in medium after 1 week of HORCs with PDGF-AA, PDGF-AB and VEGF at concentration of 50ng/ml (mean±SEM, n=6). Results expressed as % of control, * $p<0.05$ vs control. Student's t-test was used.

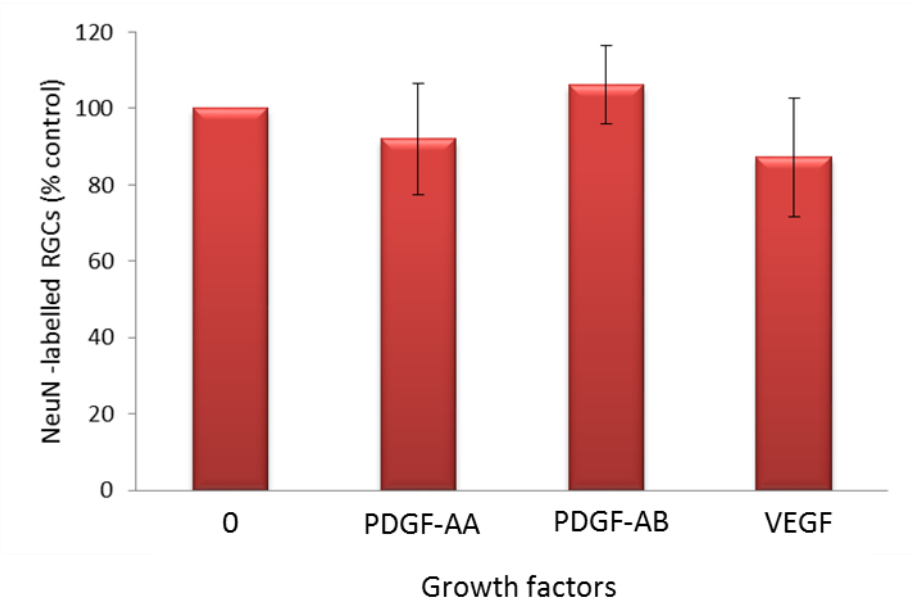


Figure 31: NeuN-labelled RGC count in HORCs after 1 week of HORCs with PDGF-AA, PDGF-AB and VEGF at concentration of 50ng/ml (mean±SEM, n=6). Results expressed as % of control. Student's t-test was used.

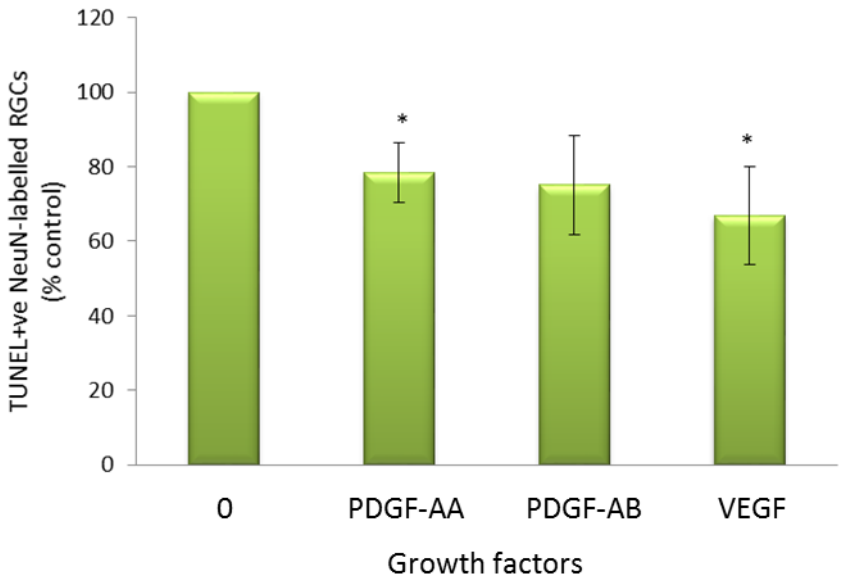


Figure 32: A proportion of apoptotic RGCs after 1 week of HORCs with PDGF-AA, PDGF-AB and VEGF at concentration of 50ng/ml (mean±SEM, n=6). Results expressed as % of control, * $p<0.05$ vs control. Student's t-test was used.

Discussion

The aim of the work presented in this chapter was to determine neurotrophic effect of specific MSC-derived growth factors that can potentially prolong survival of RGC in HORCs. Four growth factors were tested that are known to be secreted by bmMSCs: LIF, PDGF-AA, PDGF-AB and VEGF. Both PDGF-AA and VEGF conferred significant neurotrophic effects on RGCs in the long-term human retinal explant culture. In a recently published paper, Johnson and colleagues (2014) have characterised the human bmMSC secretome and established that LIF, PDGF-AA and PDGF-AB were secreted at significantly higher levels by human MSCs in comparison to human fibroblasts, whereas VEGF was found to be present in culture media of both cell types (Johnson *et*

al. 2014). The authors tested the effect of LIF, PDGF-AA and PDGF-AB on RGC survival in long-term rat retinal explants. There was a robust increase in the level of RGC survival after 1 week in rat retinal explant cultures treated with PDGF-AA and PDGF-AB, whereas LIF failed to attenuate RGC fate (Johnson *et al.* 2014). These findings are in agreement with the results using HORCs. It has also been demonstrated using *in vivo* rodent models that LIF can promote regeneration of nerves after mechanical injury (Cheema *et al.* 1994, Dowsing *et al.* 2000), in motor neurone disease (Ikeda *et al.* 1995) and amyotrophic lateral sclerosis (Azari *et al.* 2001), but, in a clinical randomised trial on patients with cancer, LIF has failed to prevent chemotherapy induced neuropathy (Davis *et al.* 2005). Despite the data presented above, the lack of trophic effect on RGCs by LIF is still surprising since there is rather strong evidence using *in vivo* animal models (Blesch *et al.* 1999, Johnson *et al.* 2011, Suzuki *et al.* 2005) and *in vitro* studies using cell cultures (Han *et al.* 2013, Majumder *et al.* 2012) demonstrating that LIF promotes survival of neurons. Majumder and colleagues (2012) have shown a significant increase in survival of human embryonic stem cell derived neuronal cells in cultures with added 10ng/ml of LIF (Majumder *et al.* 2012). Han and colleagues (2013) have identified LIF as neuroptotective factor against oxidative stress in neuronally differentiated PS12 cells in culture (Han *et al.* 2013). The concentrations tested by authors were at least 10 and 20 times smaller (0.5 and 1ng/ml) than concentrations tested in the experiments reported in this chapter. The effect of protection against apoptosis was dose-dependent and authors suggested that higher doses of LIF would most likely exert even more prominent level of

neuroprotection (Han *et al.* 2013). However, our results using HORCs do not support this suggestion demonstrating a biphasic effect to increase concentrations. In addition, localization of LIFR in the retina has also been investigated. Sarup and colleagues (2004) have demonstrated co-localization of LIFR and fluorogold pre-labelled RGCs, as well as Müller cells before and after optic nerve transaction in the rat retina (Sarup *et al.* 2004). There was no data regarding LIFR presence in the photoreceptor layer. It appears, however, that the neuroprotective effect of LIF in the retina is mainly conferred to photoreceptors (Burgi *et al.* 2009, Chollangi *et al.* 2009, Joly *et al.* 2008, Von Toerne *et al.* 2014). Neuroprotective effect of LIF on photoreceptors was demonstrated *in vitro* using retinal explants from both mice with congenital progressive photoreceptor degeneration and porcine retinas (Von Toerne *et al.* 2014), as well as an *in vivo* mouse model of retinitis pigmentosa (Joly *et al.* 2008) and following light-induced oxidative stress (Burgi *et al.* 2009, Chollangi *et al.* 2009). Although HORCs were extensively characterized as a model for studying RGC degeneration and the levels of apoptosis in the other retinal nuclear layers were not routinely quantified, it would be useful to use the human retina explants to investigate the fate of other cell types in the retinal tissue. Because after 1 week in culture, there was not much apoptosis observed in the ONL of HORCs (figure 28), it is possible to conclude that HORCs might need to be cultured for a longer period to establish the effect of LIF on other cell types in the human retina.

In contrast to LIF, the results presented in this chapter have demonstrated neurotrophic effects of both VEGF and PDGF-AA on RGC survival in HORCs.

A reduction in the number of apoptotic RGCs was also noticed in HORCs with added PDGF-AB, however the effect was not significant. The understanding of the role of VEGF as a critical survival factor for not only vascular endothelium (Gerber *et al.* 1998), as had been thought initially, but for other cell types, including neurons in both the central and peripheral nervous systems (Khaibullina *et al.* 2004, Ma *et al.* 2011, Oosthuyse *et al.* 2001, Rosenstein *et al.* 2003), has expanded dramatically over recent years. Localisation of VEGF and its receptors in the retina has been demonstrated in monkeys and rats, where *VEGF* mRNA has been localised to the INL and the RPE, and to a lesser extent to the ONL (Kim *et al.* 1999). It has also been shown using the same species that RGCs express both VEGFR1 and VEGFR2, with VEGFR2 being the most abundant receptor (Foxton *et al.* 2013, Kim *et al.* 1999). The expression of VEGF receptors was also demonstrated in the retinal glia and amacrine cells (Famiglietti *et al.* 2003) highlighting the complexity of the VEGF neurotrophic activity in the retinal tissue. The importance of VEGF for survival of not only RGCs, but also other cell types in the retina has been demonstrated by many studies. The neuroprotecive effect of VEGF to RGCs has recently been demonstrated using an *in vivo* rat model of diabetic retinopathy, where inhibition of VEGF caused a significant loss of RGCs (Park *et al.* 2014). Foxton and colleagues (2013) have demonstrated a robust neuroprotective effect of VEGF on RGC survival using an *in vitro* isolated rat RGC model, as well as an *in vivo* rat model of ocular hypertension. The effect was found to be mediated via phosphorylation of PI3K/Akt anti-apoptotic pathway (Foxton *et al.* 2013). A dose-dependent effect of exogenous VEGF to

rescue RGCs has also been shown *in vivo* using a rat model of ischaemia/reperfusion, and *in vitro* on retinal explants as a rat model of retinal degeneration (Nishijima *et al.* 2007). In addition, the importance of endogenous VEGF to Müller, bipolar, amacrine cells and photoreceptor cells survival has been demonstrated *in vitro* (Saint-Geniez *et al.* 2008), as well as *in vivo* using rat models of ischaemia/reperfusion (Nishijima *et al.* 2007) and diabetic retinopathy (Park *et al.* 2014). There is, therefore, a large body of data to suggest that VEGF is an important survival factor for RGCs. The results presented in this chapter are the first that demonstrate the neurotrophic action of VEGF to RGCs in the human retina.

Regarding PDGFs, Johnson and colleagues (2014) were the first to identify a key role of PDGF signalling in the mechanism of MSC-mediated neuroprotection. The authors demonstrated that both PDGF-AA and PDGF-AB exerted robust neuroprotective effects on RGCs *in vitro* using rat retinal explant cultures, and on RGC axons *in vivo* using the rat model of ocular hypertension. The mechanism of neuroprotection was shown to be PI3 kinase-dependent (Johnson *et al.* 2014). The results are not surprising since the expression of PDGFs and their receptors in the retina and the ON has been extensively investigated using rodent (Biswas *et al.* 2008, Mekada *et al.* 1998, Mudhar *et al.* 1993) and human retinas (Bozanic *et al.* 2006). In the rodent retina, the expression of PDGF-AA was found to be localised to the GCL and amacrine cells, whereas the expression of PDGFR α was localised to retinal and optic nerve astrocytes (Mudhar *et al.* 1993). The expression of PDGFR-α and-β was also demonstrated in the GCL, Müller cells and retinal vasculature

of rats, activation of which led to downstream signalling through PI3K/Akt pathway (Biswas *et al.* 2008). Another study also showed immunoreactivity and mRNA expression of *PDGF-B* chain in the GCL and the NFL of the rat retina, with a decline in the number of PDGF-B positive cells after the ON transection indicating a potential neurotrophic role of this factor in the RGC survival (Mekada *et al.* 1998). A critical role of PDGFs in regulation of retinal cell proliferation, differentiation, and survival during eye development was demonstrated in study using human embryos, where the expression of PDGFR-β was localized to both the pigmented and neural retina (Bozanic *et al.* 2006). In addition, the neuroprotective effects of another member of the PDGF family, PDGF-CC, was also reported in the comprehensive study by Tang and colleagues (2010) using different models of neurodegeneration, including optic nerve crush, oxidative stress and NMDA–induced apoptosis. The authors demonstrated a critical role of PDGF-CC in neuronal survival in both the retina and the brain through glycogen synthase kinase 3 β signalling (GSK3β) (Tang *et al.* 2010). It follows from the results presented above that both VEGF and PDGFs play a crucial role in RGC survival.

Importantly, both VEGF and PDGF are also known to exert a coordinated ability to regulate vessel development, where VEGF facilitates new vessel growth while PDGF stabilises vessel maturation by supporting pericyte–endothelial interactions (Benjamin *et al.* 1998, Bergers *et al.* 2003, Erber *et al.* 2004, Gendron 1999). These findings raise concerns about the use of anti-VEGF agents in patients with glaucoma for treatment of ocular neovascularization (Chong 2012) that are extensively used in current clinic

practice (Gragoudas *et al.* 2004, Krizova *et al.* 2014, Rosenfeld *et al.* 2011, Stepien *et al.* 2009, Stewart 2011). It has been demonstrated using an *in vivo* rat model, that repeated intravitreal injections of anti-VEGF bevacizumab caused RGC loss via apoptosis (Romano *et al.* 2012). Because the efficacy of a VEGF-A blockade was shown to diminish over time (Jo *et al.* 2006) and some patients are failing to respond to anti-VEGF treatment (Brown *et al.* 2009, Cohen *et al.* 2012, Heier *et al.* 2006), there is increasing interest in a specific targeting of both VEGF and PDGF signalling pathways that appear to be more effective at preventing and regressing pathological ocular neovascularization than targeting VEGF-A signalling alone (Jo *et al.* 2006, Liegl *et al.* 2014, Mendel *et al.* 2003, Takahashi *et al.* 2009). The rapid development of anti-angiogenic treatment in turn highlight the importance of further research in the effect of dual VEGF/PDGF inhibition on RGC survival, where long-term HORCs could become an invaluable resource for further investigations of such effects.

In summary, the results presented in this chapter have identified specific bmMSC-related growth factors, VEGF and PDGF-AA, which can prolong RGC survival in a human model of RGC degeneration.

CHAPTER 5

HISTONE DEACETYLASE REGULATION OF GENE EXPRESSION IN HUMAN ORGANOTYPIC RETINAL CULTURES

Introduction

Having established that specific MSC-derived growth factors possess the potential to prolong RGC survival in HORCs, the way to attenuate the expression of RGC gene markers in order to extend the period of the HORC use for neuroprotective studies on RGC gene level has also been explored.

In general, an early event in cell apoptosis is silencing of normal gene expression, including anti-apoptotic genes (Soto *et al.* 2008, Yang *et al.* 2007), with subsequent activation of gene transcription required for apoptosis (Libby *et al.* 2005) and involved in activation of the caspase cascade (Hengartner 2000). There is mounting evidence that apoptosis is the main mechanism of RGC death in glaucoma (Hanninen *et al.* 2002, Johnson *et al.* 2000, McKinnon *et al.* 2002, Quigley *et al.* 1995). It has also been found that the changes in the pattern of gene expression in experimental glaucoma occurred before detectable RGC loss (Ahmed *et al.* 2004, Buckingham *et al.* 2008, Huang *et al.* 2006, Schlamp *et al.* 2001, Soto *et al.* 2008). One of the major mechanisms involved in the control of gene activity is epigenetic regulation of chromatin folding and organization through histone modifications (Jaenisch & Bird 2003, Ramakrishnan 1997). Other mechanisms of epigenetic regulation include DNA methylation (Issa 2000, Lillycrop *et al.* 2014), chromatin remodelling (Cairns

2009, Lorch *et al.* 1999), and noncoding regulatory RNAs, for example microRNA and long noncoding RNA (Ge & Lin 2014). The fundamental subunit of chromatin is the nucleosome, which is comprised of eight positively charged histone proteins, as a cylindrical core, with negatively charged 146bp of DNA wrapped in 1 ¾ turns around them (Arents *et al.* 1991, Luger *et al.* 1997). Because nucleosomes control the accessibility of promoter DNA by preventing the binding of transcription factors to this region (Richmond & Davey 2003, Segal *et al.* 2006), both position and density of nucleosomes are strongly linked to the specific DNA sequence and mainly represented in a pattern of nucleosome-depleted regions and nucleosome-enriched areas located at the gene promoters close to the transcription start sites (Lee *et al.* 2007, Mavrich *et al.* 2008). The chain of nucleosomes is then wrapped into a spiral, called a solenoid, which further condenses and folds to form a chromosome. In this condensed form (heterochromatin) genes are silenced because they are inaccessible to DNA binding proteins for gene transcription. Each nucleosome core contains eight histone proteins, consisting of two copies of four histones: H2A, H2B, H3, and H4. There is an additional, unpaired H1 histone associated with each nucleosome that promote organization of nucleosomes into a higher-order structure (Ramakrishnan 1997). Each of those histones has the N-terminal tail positioned outside of the nucleosome core. A wide variety of post-translational modifications of these histone tails influence the change in chromatin architecture and include, but are not limited to, lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination (Jenuwein & Allis 2001, Ramakrishnan 1997, Vaquero *et*

al. 2003). For example, acetylation of ε-amino groups of lysine residues in the positively charged N-terminal of histone tails interferes with their binding to DNA, thus allowing an opening of the chromatin configuration and enabling access to DNA-binding proteins for gene transcription (Rosato & Grant 2005). Acetylation is believed to have the most direct effect on chromatin structure, and is dependent upon two families of proteins: histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Shahbazian & Grunstein 2007) (figure 33). Deacetylation of histones causes a compaction of chromatin that in turn leads to gene silencing (Grant & Dai 2012, Rosato & Grant 2005). Inhibition of deacetylation promotes the active, de-condensed chromatin conformation (figure 33), and, therefore, provides the access of transcriptional factors to DNA for gene transcription.

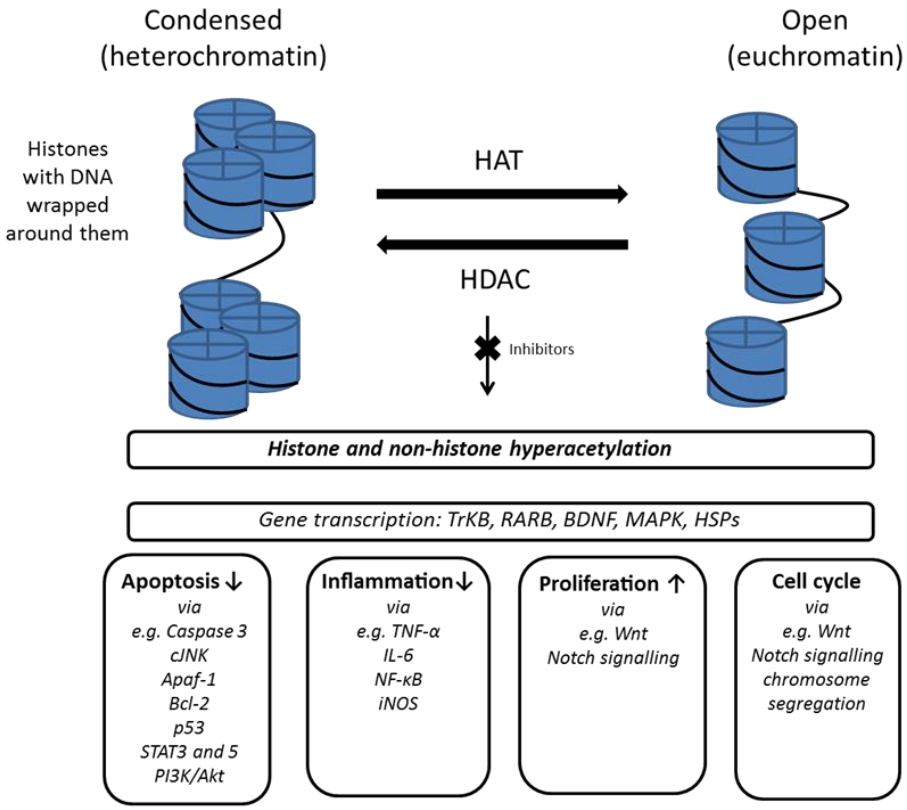


Figure 33: Schematic representation of histone deacetylase inhibitors (HDACIs) induced chromatin transition and some associated downstream effects. Inhibition of HDACs by HDACIs allows hyperacetylation of histone and non-histone proteins, which leads to changes in several physiological and pathological processes, including apoptosis, cell cycle progression, inflammation and proliferation, inflammation. HAT=histone acetyl transferase, HDAC=histone deacetylase, TrKB=tropomyosin-related kinase B, RARB= retinoic acid receptor β, BDNF=brain-derived neurotrophic factor, MAPK =mitogen-activated protein kinases, HSPs=heat shock proteins, cJNK=c-Jun N-terminal kinase, Apaf-1=apoptotic-protease-activating factor-1, Bcl-2=B-cell lymphoma 2, STAT3 and 5= signal transducer and activator of transcription, PI3K/Akt=phosphatidylinositol-3-kinase/Akt pathway, TNF-α=tumour necrosis factor-α, IL-6=interleukin-6, NF-kB=nuclear factor kappa-light-chain-enhancer of activated B cells, iNOS=inducible nitric-oxide synthase.

HDACs are the products of 18 genes and are divided into four classes. Class I (HDACs 1, 2, 3 and 8), class IIA (HDACs 4, 5, 7, and 9), class IIB (HDACs 6 and 10) and IV (HDAC 11) are zinc-dependent enzymes, whereas the class III enzymes (sirtuins) are zinc-independent, but are dependent on nicotinamide

adenine dinucleotide (NAD^+) (Rosato & Grant 2005). Class I, II, and IV are referred to as "classical" HDACs (Witt *et al.* 2009). Although histones were the first substrates described for these enzymes (Hebbes *et al.* 1988, Lee *et al.* 1993), the activity of diverse non-histone proteins is also modified by HATs and HDACs, for example, proteins involved in the regulation of signal transducer and activator of transcription (STAT) 3 and 5 (Pang *et al.* 2009, Rascle *et al.* 2003), c-Jun N-terminal kinase (cJNK) activation (Dai *et al.* 2010, Vrana *et al.* 1999), and angiogenesis regulation through alternation of vascular growth factors signalling (Deroanne *et al.* 2002, Qian *et al.* 2004). It was found that HDAC6 mediated acetylation controls the function of heat shock protein 90 (Bali *et al.* 2005, Kovacs *et al.* 2005, Murphy *et al.* 2005) and αtubulin (Ryhanen *et al.* 2011, Zhang *et al.* 2003). It has been reported that HDAC enzymes are also involved in control of gene transcription (Walkinshaw *et al.* 2008, Wang *et al.* 2001), cell-cycle progression and cell differentiation (Jamaladdin *et al.* 2014, Kim *et al.* 2002, Wang *et al.* 2001), and apoptosis (Juan *et al.* 2000, Luo *et al.* 2000, Pelzel *et al.* 2010). Knockdown of HDAC1 and 2 genes in embryonic stem cells caused cell death associated with abnormal cell mitosis and an increase in a number of chromosome segregation defects (Jamaladdin et al. 2014). Pelzel and colleagues (2010) have shown that apoptosis of neurons was associated with an increase in expression of HDAC3 with corresponding suppression of histone H4 acetylation (Pelzel *et al.* 2010). Overexpression of class 1 HDACs has been extensively demonstrated in cancer cells, especially of the gastrointestinal system (Choi *et al.* 2001, Wilson *et al.* 2006), and is associated with advanced disease staging and poor prognosis

(Weichert *et al.* 2008). It has also been demonstrated that HDAC1 can directly deacetylate and inhibit activity of the tumour suppressor factor p53 (Juan *et al.* 2000, Luo *et al.* 2000).

Various natural and synthetic chemical compounds possess the ability to inhibit HDACs and are classified based on their chemical structure, for example, short-chain fatty acids (valproic acid (VPA) and sodium phenyl butyrate(SB)), hydroxamic acids (trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA, Vorinostat), cyclic peptides (romidepsin, apicidin, cyclic hydroxamic acid-containing peptides (CHAPS), and trapoxin), benzamides (entinostat (MS-275) and tacedinaline (CI-994)), ketones (trifluoromethyl ketone) and miscellaneous compounds (MGCD-0103 and nicotinamide) (Konsoula & Barile 2012, Tang *et al.* 2013). From those HDACIs, vorinostat (Zolinza™, Merck) and romidepsin (Istodax®, Celgene) have already been approved for the treatment of patients with peripheral and cutaneous T-cell lymphomas (Watanabe 2010). Because of a wide spectrum of HDAC activity, there is currently considerable interest in HDAC inhibitors (HDACIs) in relation to numerous pathological processes and diseases, including cancer (Bose *et al.* 2014, Watanabe 2010), interstitial fibrosis (Davies *et al.* 2012, Nural-Guvener *et al.* 2014), inflammation (Leoni *et al.* 2002, Lin *et al.* 2007), septic shock (Zhang *et al.* 2010a) and diabetes (Christensen *et al.* 2011, Hara *et al.* 2014), as well as immunomodulation after organ transplantation (Edens *et al.* 2006). However, the understanding of the complexity of HDACIs pleiotropic mechanisms of action and associated with it effects is still evolving (Bose *et al.* 2014, Ciarlo *et al.* 2013).

Importantly, the expression of HDACs has been investigated in the rat brain and found to be localized to neurones and glial cells (Broide *et al.* 2007). Moreover, inhibition of HDAC has also been shown to be protective in several models of neurodegenerative disease by ameliorating transcriptional dysfunctions in Alzheimer's and Parkinson's diseases, as well as glaucoma. Multiple studies have reported on a neuroprotective action of TSA (Pelzel *et al.* 2010, Pelzel *et al.* 2012, Seo *et al.* 2013, Wu *et al.* 2008), a product of fermentation from *Streptomyces,* a gram-positive *Actinobacteria*. TSA is known to inhibit both class I and class II HDACs and, based on its chemical structure, belongs to hydroxamic acid group of HDACIs. Originally, TSA was used as an anti-fungal agent, but later it was discovered to have a potent anti-proliferation property on cancer cells (Yoshida *et al.* 1990). For example, neurotrophic and neuroprotective properties of TSA, SB and VPA have been demonstrated using nigrostriatal dopaminergic neurons in rat neuronal–glial cultures through up-regulation of GDNF and BDNF mRNA expression in rat primary cortical astrocyte cultures caused, at least in part, by hyperacetylation of histones in the promoter region of genes (Wu *et al.* 2008). Moreover, an ability of VPA to down regulate the release of pro-inflammatory factors by microglia has also been investigated using lipopolysaccharide (LPS) and 1-methyl-4-phenylpyridinium (MPP+) induced models of neurotoxicity in rat primary mesencephalic neuron-glia cultures (Chen *et al.* 2006). Recently, a role of TSA has also been investigated in calcium-induced neuronal cell death. The authors demonstrated an inhibition of calpain activity which was partly mediated by an increase in calpastatin (the endogenous inhibitor of calpain)

expression via histone hyperacetylation within the calpastatin gene promoter region (Seo *et al.* 2013). In relation to glaucoma, transcriptional down-regulation of the RGC gene makers expression has been shown to be an early event in animal models of glaucomatous RGC degeneration (Ahmed *et al.* 2004, Schlamp *et al.* 2001, Weishaupt *et al.* 2005). Pelzel and colleagues (2010) have demonstrated that a preservation of RGC-specific gene expression, as well as an alteration of RGC loss was associated with TSA treatment using an *in vivo* mice model of optic nerve crush (Pelzel *et al.* 2010).

The aim of the work presented in this chapter was to determine effects of the HDAC inhibitor TSA on expression of retinal cell marker genes in the human retina and to investigate a possible neuroprotective effect of TSA on human RGCs in HORCs.

Results

TSA increased total RNA in HORCs

As a first step in examining the effect of TSA on gene expression in the human retina, total RNA yield from HORCs treated with TSA was measured after 12, 24 and 48 hours in culture. In control HORCs, there was a progressive loss of total RNA with time (figure 34). In cultures with TSA, a dose and time-dependent effect on total RNA loss was observed (figure 35). The most pronounced effect of TSA on the amount of total RNA was noticed after 48 hours in culture with 1µM and 10µM TSA. After 48 hours in culture, TSA at all tested concentrations (0.1, 1 and 10µM) inhibited the loss of total RNA.

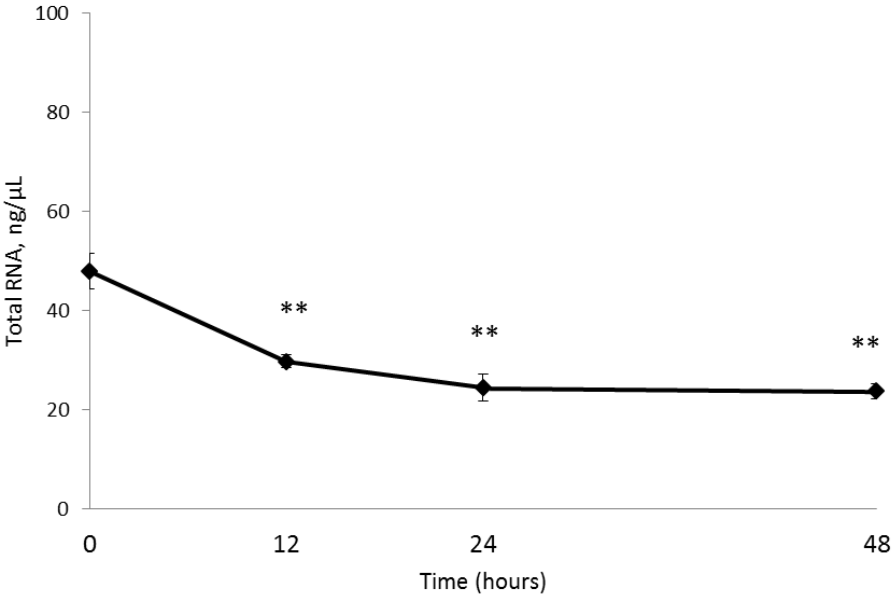


Figure 34: Total RNA loss in HORCs cultured for 12, 24 and 48 hours. RNA concentration is expressed as ng/µl (mean±SEM, n=4-12), **p<0.005 vs t=0. Student's t-test was used.

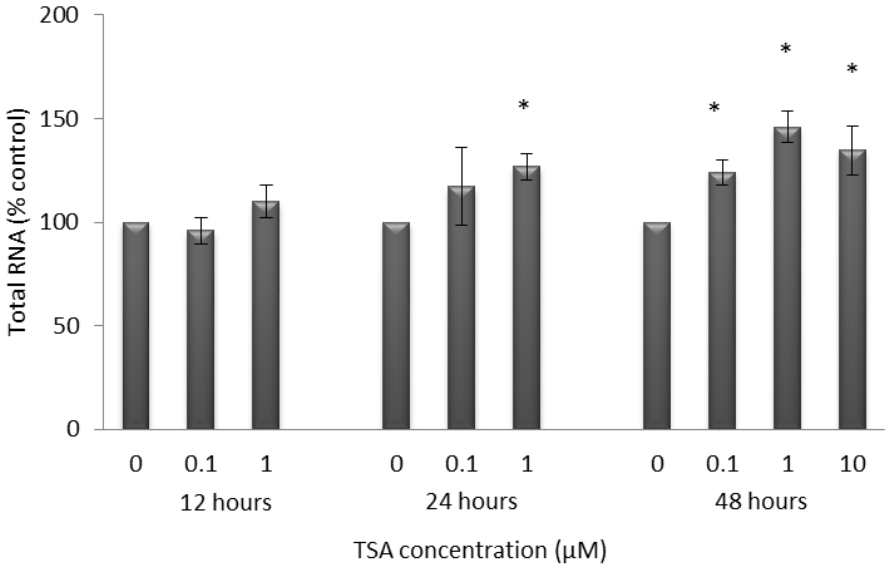
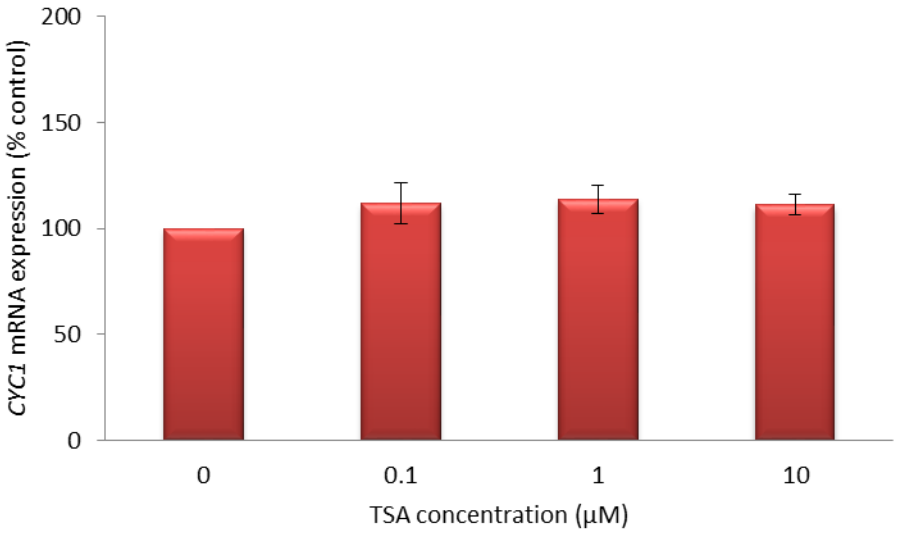


Figure 35: Total RNA in HORCs with added TSA (0.1, 1 and 10µM) and controls cultured for 12, 24 and 48 hours (mean±SEM, n=4-12). RNA concentration is expressed as % controls at 12, 24 and 48 hours respectively,*p≤0.05 vs control. Student's t-test was used.

TSA caused no change in expression of housekeeping genes

Because the effect of TSA on total RNA was the most pronounced after 48 hours, the effect on expression of retinal cell markers in HORCs was investigated at this time point. First of all, it was important to identify if there were any changes in the housekeeping gene expression. Housekeeping genes are examples of constitutive genes that are constantly switched on and control essential proteins important to cell survival (Tirosh & Barkai 2008). Expression of cytochrome c-1 (*CYC1*) and topoisomerase 1 (*TOP1*) mRNAs was shown to be the most stable in human retinal explants (Niyadurupola *et al.* 2011), thereby allowing them to be utilized as housekeeping genes for normalization of QRT-PCR results. As can be seen in figure 36, expression for both mRNAs was relatively stable in HORCs treated with 0.1, 1 and 10µM TSA after 48 hours in culture.

A



B

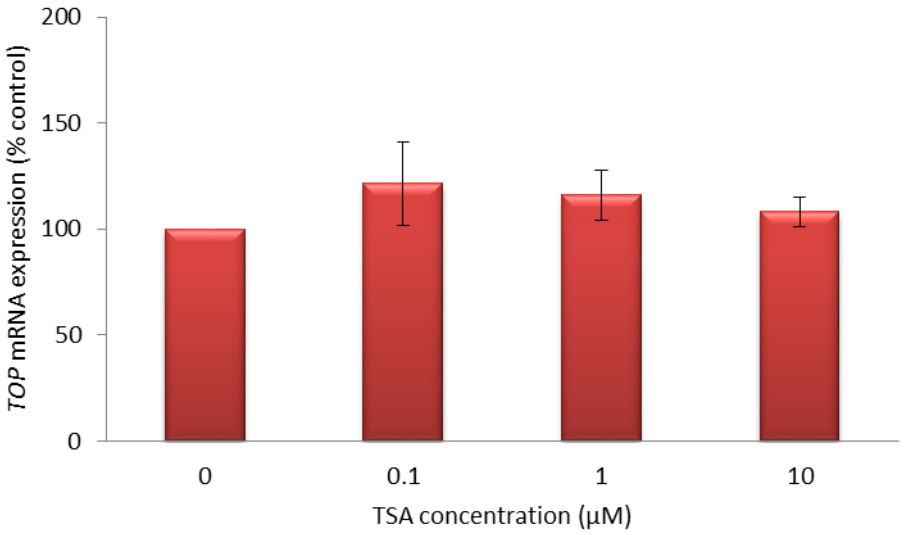


Figure 36: Expression of the housekeeping genes (A) *CYC1* and (B) *TOP1* in HORCs cultured with TSA (0.1, 1 and 10µM) for 48 hours (mean±SEM, n=4). Gene expression is presented as % control. Student's t-test was used.

TSA up-regulated expression of retinal ganglion cell markers genes

Expression of selected RGC-related marker genes was measured using quantitative PCR method. All selected genes were known to be highly specific for retinal ganglion cells (Barnstable & Drager 1984, Kim *et al.* 2009, Ma 2013, Nadal-Nicolas *et al.* 2009) and widely used in many studies (Buckingham *et al.* 2008, Niyadurupola *et al.* 2011, Pacal & Bremner 2014, Schlamp *et al.* 2001, Weishaupt *et al.* 2005). *Thy1* is a neuronal cell surface glycoprotein which label RGC somata and axons (Barnstable & Drager 1984). THY1 antibodies were found to be the most effective in obtaining purified RGC cultures via immunopanning (Barres *et al.* 1988, Winzeler & Wang 2013, Zhang *et al.* 2010b). Expression of *Thy1* as a marker for RGCs is extensively used in retinal degenerative disease research (Niyadurupola *et al.* 2013, Pelzel *et al.* 2010, Schlamp *et al.* 2001). As can be seen in figure 37, *Thy1* showed a dose-dependent increase in expression in HORCs with 10µM TSA, giving an approximate 60% increase compared to that in controls ($p<0.05$, $n=4$).

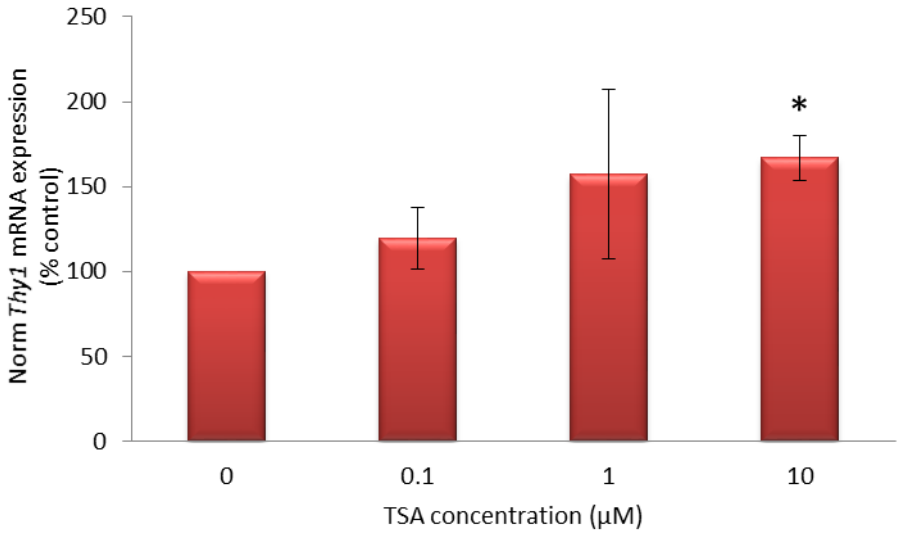
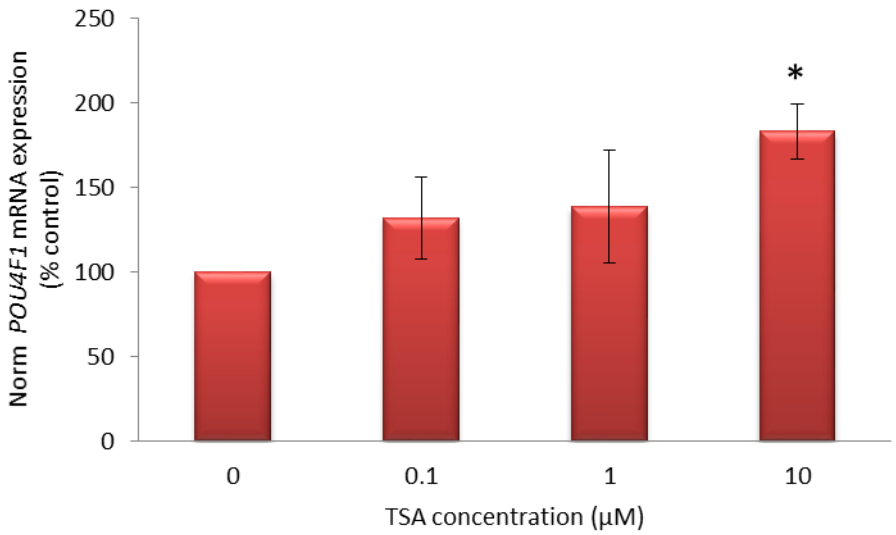


Figure 37: Expression of RGC marker gene *Thy1* in HORCs with TSA at concentrations of 0.1, 1 and 10µM for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and presented as % control,*p≤0.05 vs control. Student's t-test was used.

Since there was an increase in expression of the RGC marker Thy1, the expression of other marker genes for RGCs was also measured (figure 38). *Brn3a* (aka POU class 4homebox 1, *POU4F1*) is a member of *Brn3* gene family of POU domain transcription factors necessary for neuronal differentiation (Nadal-Nicolas *et al.* 2009, Weishaupt *et al.* 2005). A down-regulation of *Brn3a* mRNA expression was demonstrated after an acute ON injury in rats (Weishaupt *et al.* 2005). It was also shown that activation of *Brn3a* stimulates transcription of anti-apoptotic genes (Hohenauer *et al.* 2013, Hudson *et al.* 2004). Neuronal nuclei (NeuN) protein (aka RNA binding protein fox-1 homolog 3, *Rbfox3*) is a DNA-binding protein and is used as a specific marker for post-mitotic (mature) neurons (McKee *et al.* 2005). NeuN

was initially identified as a specific antibody for immunolabelling of neuronal cells, including RGCs (Buckingham *et al.* 2008, Diaz *et al.* 2005, Niyadurupola *et al.* 2013). Kim and colleagues (2009) identified *Rbfox3* as a gene for NeuN protein. Fox (feminizing on X) proteins are a highly conserved family of tissue-specific splicing regulators (Dredge & Jensen 2011, Kim *et al.* 2009). Both *Brn3a* (*POU4F1*) and *NeuN* (*Rbfox3*) mRNA expression increased with TSA treatment in a dose-dependent manner. A similar pattern of expression to *Thy1* mRNA was seen in HORCs with 10µM TSA for both genes. There was a significant increase of around 80% in *POU4F1* mRNA expression compared to that in controls ($p<0.05$, $n=4$). Up-regulation of *Rbfox3* mRNA was less prominent, but still significant, showing about a 30% increase ($p<0.05$, $n=4$).

A



B

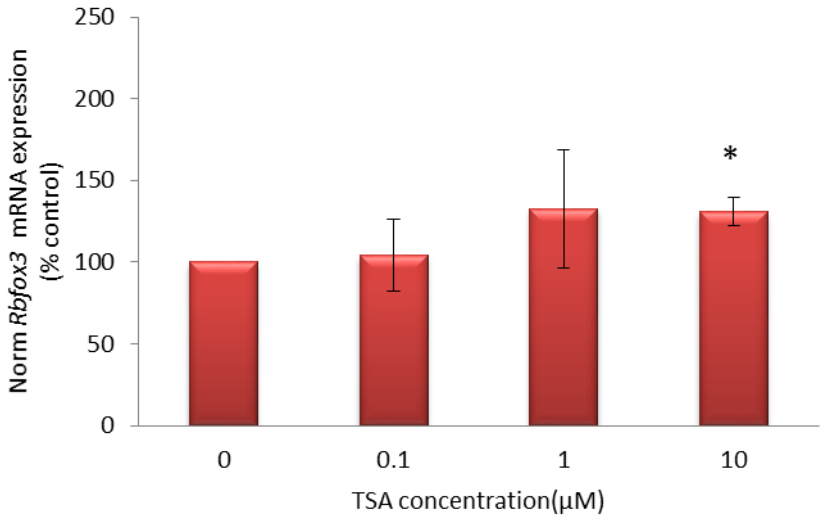


Figure 38: Expression of RGC marker genes (A) *Brn3a* (*POU4F1*) and (B) *NeuN* (*Rbfox3*) in HORCs cultured with TSA at concentrations of 0.1, 1 and 10µM for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and presented as % control, *p≤0.05 vs control. Student's t-test was used.

TSA is specific to retinal ganglion cells

To investigate whether expression of other retinal cell makers would respond to TSA treatment in the same way as RGC-related mRNAs, gene markers for photoreceptors (*RCVRN*) (Dizhoor *et al.* 1991, Wiechmann *et al.* 1994), amacrine (*ChAT*) (Haverkamp & Wassle 2000), horizontal (*CALB*) (Nakhai *et al.* 2007), and the retinal glia (*Glul*) (Chang *et al.* 2007, Derouiche & Rauen 1995, Spoerri *et al.* 1997) were measured in HORCs.

Recoverin (*RCVRN*) gene encodes for a calcium-binding protein that was originally purified from bovine rod outer segments (Dizhoor *et al.* 1991, Lambrecht & Koch 1992). A key role of recoverin is in control of visual signal transduction by an inhibition of rhodopsin kinase, an enzyme that regulate the phosphorylation of rhodopsin (Senin *et al.* 1997). Imunolabelling with anti-recoverin antibodies was localized to cone and rod photoreceptors (Dizhoor *et al.* 1991, Grunert *et al.* 1994, Johnson *et al.* 1999, Korf *et al.* 1992, Milam *et al.* 1993, Wiechmann *et al.* 1994, Wiechmann & Hammarback 1993), as well as ON and OFF cone bipolar cells (Gunhan-Agar *et al.* 2000, Milam *et al.* 1993, Miller *et al.* 1999). In HORCs, the expression of *RCVRN* mRNA was very similar to controls at all tested concentrations (figure 39).

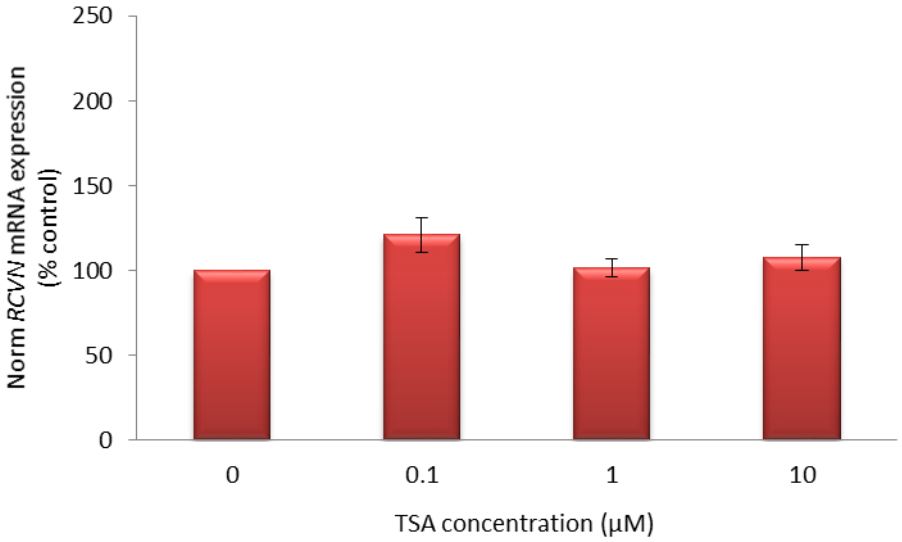


Figure 39: Expression of recoverin (*RCVN*) mRNA as a marker for photoreceptor cells in HORCs with 0.1, 1 and 10µM TSA for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and presented as % control. Student's t-test was used.

Calbindin (CALB) is one of the major calcium-binding proteins that buffer the Ca^{2+} level and involved in the Ca^{2+} transport (Baimbridge *et al.* 1992). CALB was demonstrated to be a useful marker for horizontal cells in the retinas of monkeys and rats (Chu *et al.* 1993, Rohrenbeck *et al.* 1987, Zheng *et al.* 2012). It was also shown that ganglion cells in the mouse retina are also immunoreactive for calbindin.(Haverkamp & Wassle 2000). It was also shown *in vivo* that *CALB* pays a crucial protective role against apoptosis of neurones and glial cells in the central nervous system using an *CALB*-deficient mouse model (Kook *et al.* 2014). In the HORC, the expression of *CALB* mRNA with TSA at any concentrations was no different compared to that in controls (figure 40).

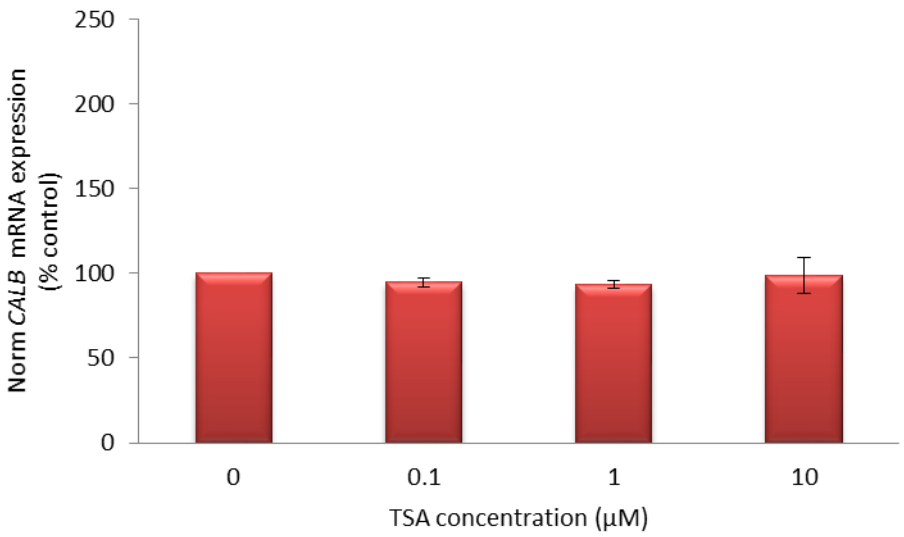


Figure 40: Expression of calbindin (*CALB*) mRNA as a marker for horizontal cells in HORCs with 0.1, 1 and 10µM TSA for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and presented as % control. Student's t-test was used.

In contrast to *RCVRN* and *CALB* that showed no significant changes in mRNA expression, there was a significant increase of about 40% in expression of choline acetyltransferase (*ChAT*), a marker for amacrine cells, in HORCs with 1µM TSA, although the trend was not dose-dependent (figure 41). ChAT is a widely used marker for immunolabelling cholinergic amacrine cells in the retinas of mice (Haverkamp & Wassle 2000, Pang *et al.* 2013), rates (Araki & Hamassaki-Britto 2000, Voigt 1986), as well as monkeys and humans (Rodieck & Marshak 1992).

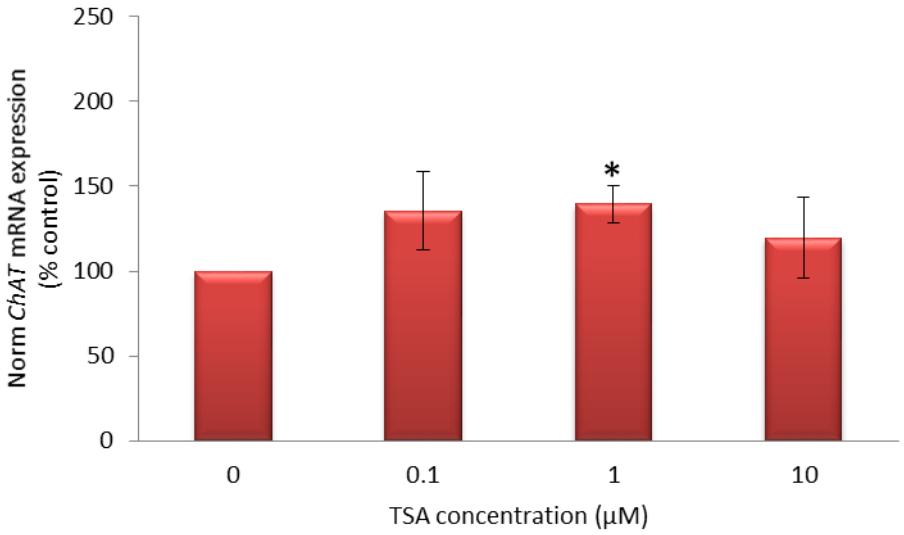


Figure 41: Expression of choline acetyltransferase (*ChAT*) mRNA as a marker for amacrine cells in HORCs with 0.1, 1 and 10µM TSA for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and presented as % control, * $p<0.05$ vs control. Student's t-test was used.

Glutamate-ammonia ligase (*Glul*) gene encodes protein that belongs to the glutamine synthase family (Wang *et al.* 1996). Glutamine synthase is an enzyme that plays a crucial role in glutamate homeostasis (Danbolt 2001). In the retina of rats and mice, glutamine synthase immunoreactivity was found to be localised to the Müller cells and astrocytes (Anlauf & Derouiche 2013, Chang *et al.* 2007, Derouiche & Rauen 1995, Haverkamp & Wassle 2000, Riepe & Norenburg 1977), where it catalyzes the synthesis of glutamine from glutamate and ammonia (Pellerin & Magistretti 1994). In HORCs, there was no difference in the level of mRNA expression of *Glul* compared to that in controls at all tested concentrations (figure 42).

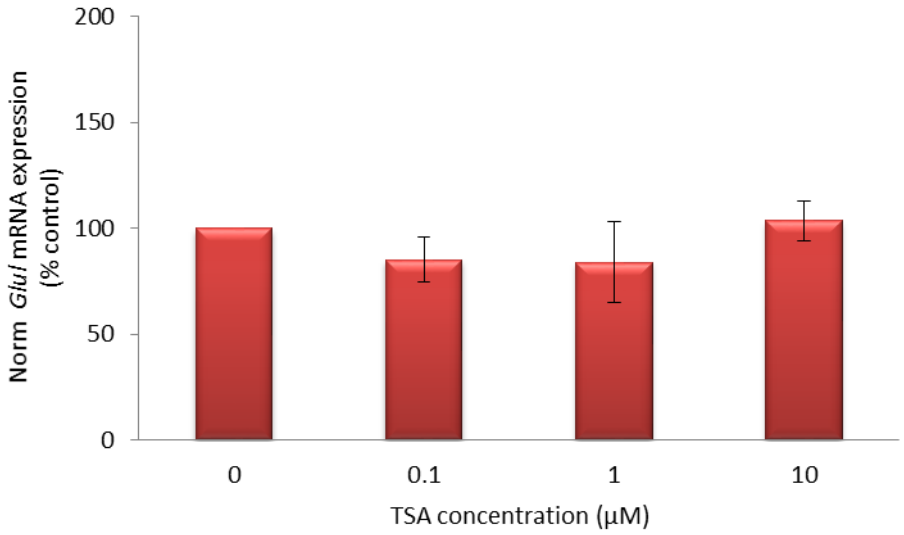


Figure 42: Expression of glutamate-ammonia ligase (*Glul*) mRNA as a marker for retinal glial cells in HORCs with TSA at concentrations of 0.1, 1 and 10µM for 48 hours (mean±SEM, n=4). Expression is normalised to *CYC1* and *TOP1* and is presented as % control. Student's t-test was used.

Expressions of glutamate–aspartate transporter (*GLAST)* and heat shock protein 70 (*HSP70* or *HSPA1B*) mRNAs were also measured in HORCs after 48 hours in culture with TSA at concentrations of 0.1, 1 and 10µM. Glutamate transporter GLAST is involved in homeostasis of extracellular glutamate. It has been demonstrated using rat and human retinas that *GLAST* is expressed by ganglion cells and the glia, (Otori *et al.* 1994), as well as horizontal, bipolar, amacrine and photoreceptor cells (Kugler & Beyer 2003). There was no difference in the level of mRNA expression of *GLAST* compared to that in controls in HORCs at all tested concentrations (figures 43).

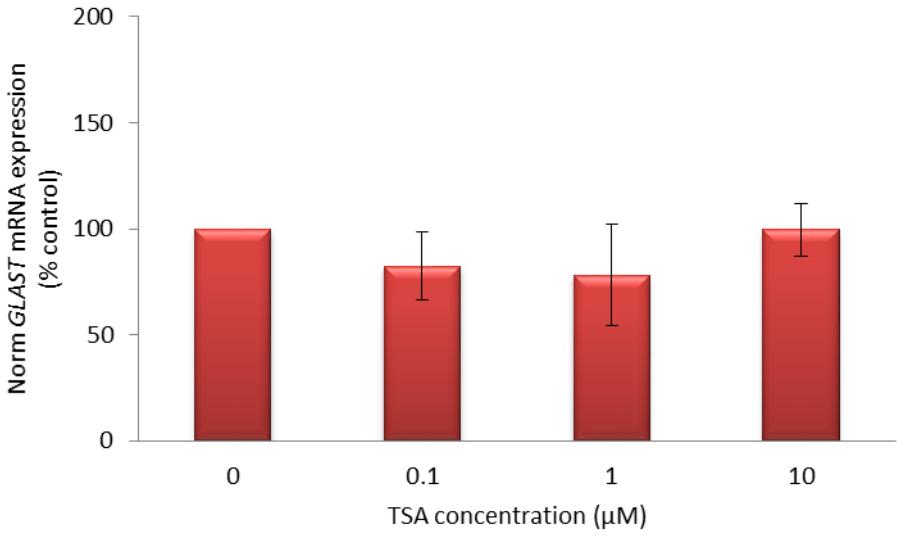


Figure 43: Expression of glutamate-aspartate transporter (*GLAST*) mRNA in HORCs cultured with TSA at concentrations of 0.1, 1 and 10µM for 48 hours (mean±SEM, n=4). Expression was normalised to *CYC1* and *TOP1* and is presented as % control. Student's t-test was used.

HSP70 is a member of the large, highly conserved family of HSPs involved in regulation of cellular homeostasis and promotion of cell survival by facilitating protein folding and degradation of abnormally folded proteins (Hartl 1996, Takayama *et al.* 2003). It was reported that HSP70 is specifically expressed in RGCs of the normal human retina (Ma 2013) and was shown to play a crucial role in RGC survival in the zebra fish retina after acute optic nerve injury (Nagashima *et al.* 2011). There was no difference in the level of mRNA expression of *HSPA1B* compared to that in controls in HORCs at all tested concentrations (figures 46). Interestingly, a dramatic increase of almost 500% in expression of *HSPA1B* mRNA was noticed in controls and HORCs with TSA compared to that in t=0 (figure 44).

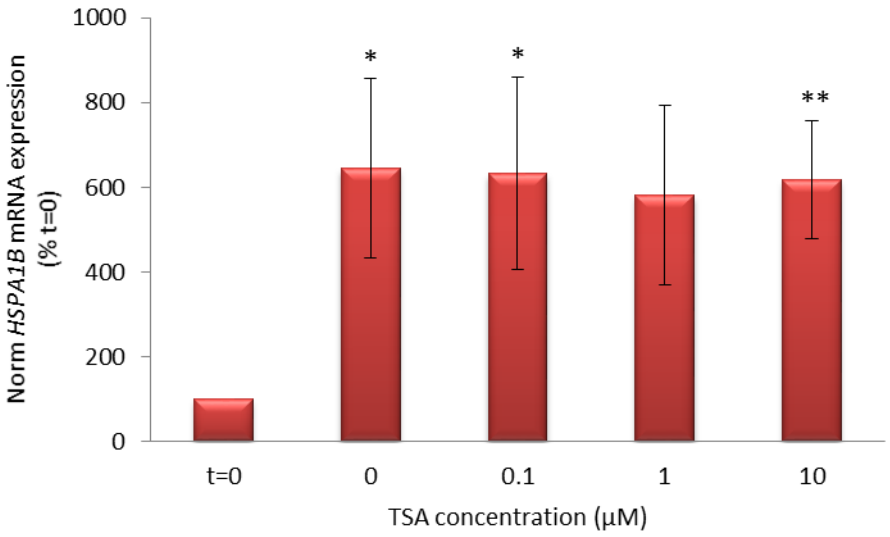


Figure 44: Expression of heat shock protein 70 (*HSPA1B*) mRNA in HORCs cultured with TSA at concentrations of 0.1, 1 and 10µM for 48 hours (mean±SEM, n=4). Expression was normalised to *CYC1* and *TOP1* and is presented as % t=0, *p≤0.05, **p<0.01 vs t=0. There was no statistically significant difference in HORCs with TSA and controls. Student's t-test was used.

TSA has no effect on RGC apoptosis in HORCs following 1 week in culture

Increased expression of RGC marker genes may indicate that TSA is promoting the survival of RGCs in culture, therefore HORCs were cultured with 0.1µM, 1µM and 10 µM TSA for 1 week. As can be seen in figure 45, nuclear cell layers were preserved under all culture conditions. LDH activity in medium was significantly higher in HORCs with 1 µM compared to that in controls (figure 46). There was no significant change in the number of NeuN-labelled RGCs (figure 47), as well as in the number of TUNEL-positive NeuN-

labelled cells, indicating that TSA did not protect RGCs from apoptosis at any of the tested TSA concentrations (figure 48).

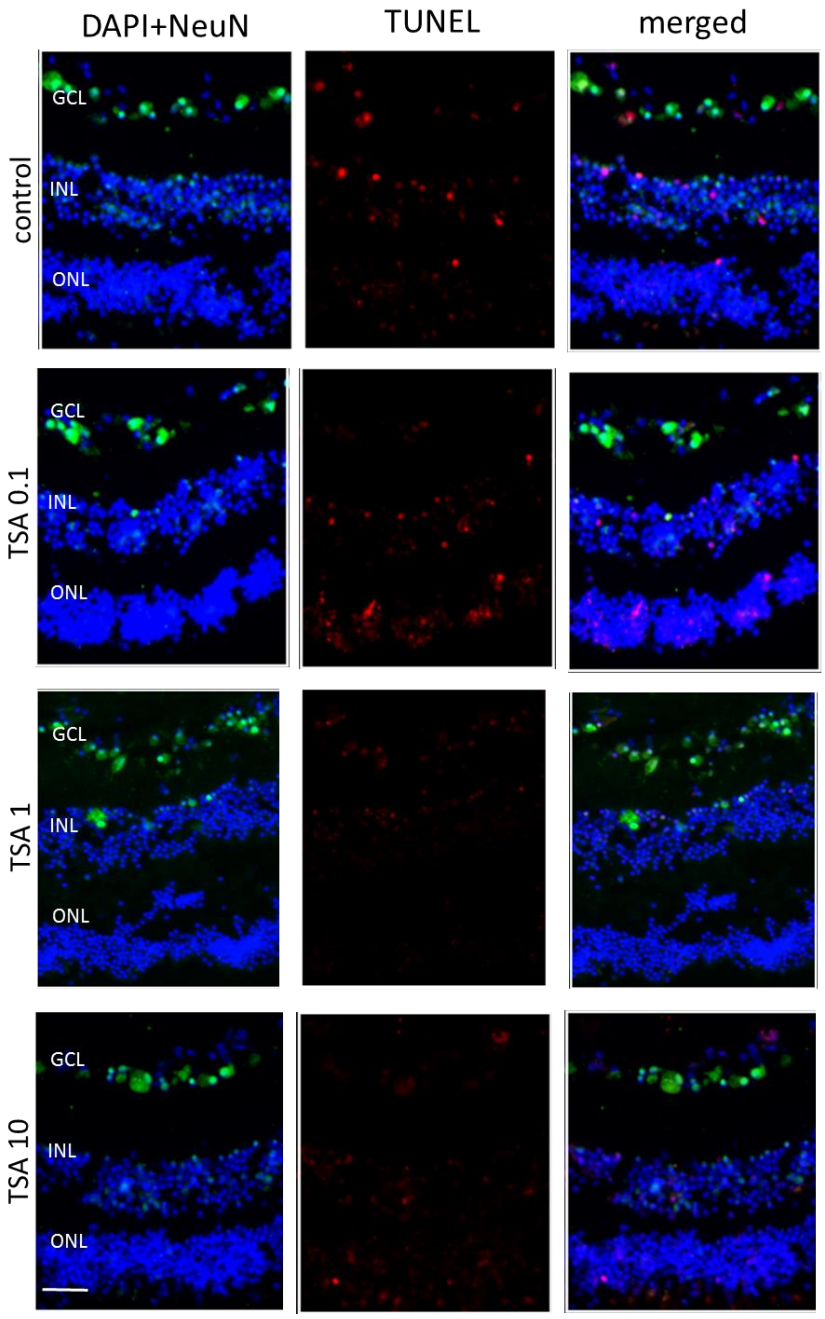


Figure 45: Representative HORC images cultured with TSA at concentrations of 0.1, 1 and 10µM for 1 week and controls. RGCs are labelled with NeuN (green), cell nuclei with DAPI (blue), apoptotic cells with TUNEL (red). Scale bar, 100µm.

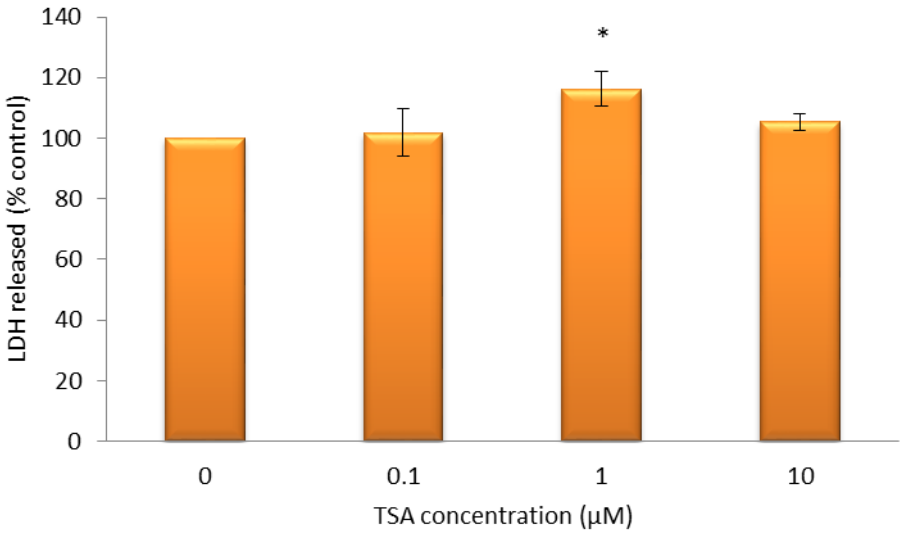


Figure 46: The relative level of LDH released in medium after 1 week HORC with 0.1µM, 1µM and 10 µM TSA and controls (mean±SEM, n=4), * $p<0.05$ vs control. Student's t-test was used.

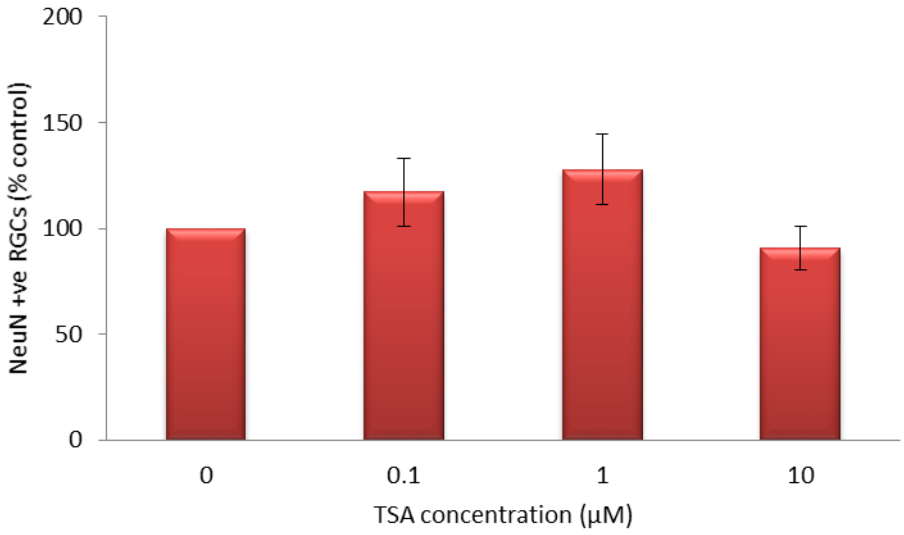


Figure 47: Number of NeuN-labelled cells in 1 week HORCs with 0.1µM, 1µM and 10 µM TSA and controls (mean±SEM, n=4). Student's t-test was used.

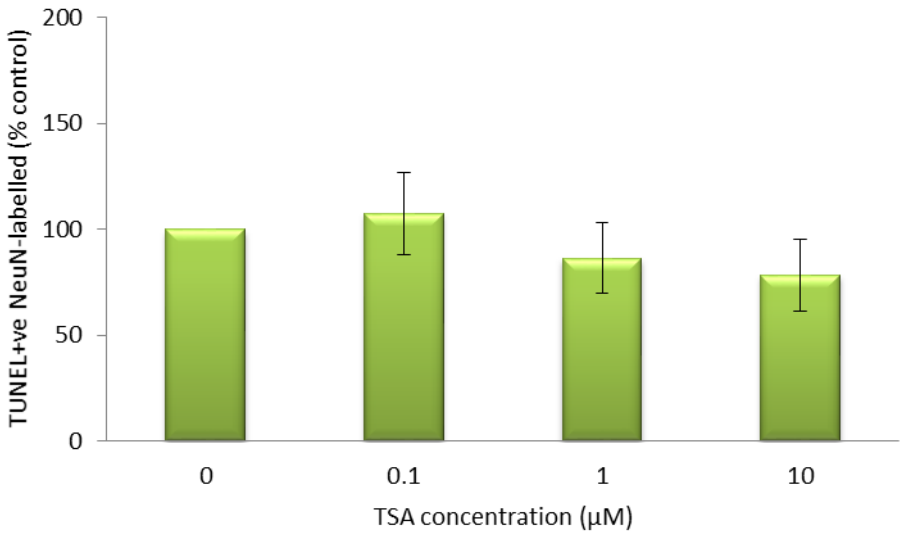


Figure 48: Percentage of TUNEL-positive NeuN-labelled RGCs in 1 week HORC with 0.1µM, 1µM and 10 µM TSA and controls (mean±SEM, n=4). Student's t-test was used.

Discussion

Down-regulation of ganglion cell-related gene expression has been documented by several studies and appeared to represent a relatively early event in the apoptotic cell death (Pelzel *et al.* 2012, Schlamp *et al.* 2001, Wang *et al.* 2010a, Weishaupt *et al.* 2005, Yang *et al.* 2007). It has also been reported that the change in the pattern of gene expression in RGCs occurs before detectable cell loss (Huang *et al.* 2006, Schlamp *et al.* 2001). Epigenetic regulation of gene expression, in particular histone hypoacetylation due to increase in activity of HDAC enzymes, has been proposed as an underlying mechanism of this phenomenon using mouse models of optic nerve crush

(Pelzel *et al.* 2010) and congenital RGC degeneration (Pelzel *et al.* 2012). In the mouse retina, Pelzel and colleagues (2010) demonstrated the presence of HDAC1-3 and 5, but not HDAC4 using both mRNA and protein analysis. After acute optic nerve injury, there was a significant and persistent increase in expression of HDAC3, which was associated with a decrease in histone H4 acetylation in promoter regions of ganglion cell marker genes, such as *Thy1*, *Brn3b*, *Fem1C* and *Nrn1*, as well as anti-apoptotic gene *BclX*, suggesting that a major role in apoptotic gene silencing is played by activation of HDAC3. Moreover, an increase in acetylation of histone H4 in promoter regions of pro-apoptotic genes *Bim* and *cJun* was also noticed. After pretreatment with TSA, a significant reduction in the RGC loss of about 50% was observed at 2 weeks post ON injury, which was associated with overexpression of acetylated histone H4, as well as an up-regulation of the RGC maker *Fem1cR3* gene (Pelzel *et al.* 2010). In addition, the effect of TSA on expression of a RGC marker *Fem1cR3* was demonstrated in a study using a congenital mouse model of secondary glaucoma. Although continuous treatment of DBA/2J mice with TSA preserved the expression of *Fem1cR3*, there was no difference in the RGC axon loss detected between treatment and control groups (Pelzel *et al.* 2012).

In the human retina, the results of the study presented in this chapter demonstrated that TSA possesses the ability to amend the loss of the RGC gene marker expression, such as *Thy1* (Barnstable & Drager 1984), *Brn3a*(*POU4F1*) and *NeuN*(*Rbfox3)*, associated with unchanged expression of retinal housekeeping genes, *TOP1* and *CYC1* (Niyadurupola *et al.* 2011). However, the reduction of TUNEL positive RGCs after 1 week in culture was not

significant, indicating that TSA did not protect RGCs. It can be suggested that preservation of RGC gene expression may support neurons in stress (dying neurons), thereby creating a beneficial environment for survival mechanisms to play the part. For example, a preserved expression of pro-survival genes might be a potential mechanism of HDACI induced neuroprotection. It has been reported that VPA and TSA exerted an ability to up-regulate the expression of anti-apoptotic *Bcl-2* gene family members (Cui *et al.* 2011, Sinn *et al.* 2007). Therefore further experiments with higher concentrations of TSA using gene markers for apoptosis, as well as TUNEL labelling, would be beneficial to clarify the neuroprotective role of TSA in experiments using HORCs. The difference in the neuroprotective effect of TSA that was found in the model of acute RGC axon injury (Pelzel *et al.* 2010) to our experiment might be explained by differences in the origin of models, in treatment timing and TSA concentrations, as well as the difference between the acute and chronic nature of the RGC degeneration. In addition, some would argue that pretreatment with TSA prior to an assault would reduce HDAC activity at the time of injury, thereby attenuating subsequent changes in gene transcription. However, Tezel and colleagues (2010) did not report on the mechanism of RGC protection, specifically on alterations in pro-apoptotic gene expression, after TSA pretreatment.

Because glutamate excitotoxicity in the retina is implicated as one of the major mechanism of glaucoma pathophysiology (Siliprandi *et al.* 1992, Sisk & Kuwabara 1985, Vorwerk *et al.* 1996), the effect of TSA on expression of mRNA for glutamate–aspartate transporter (*GLAST*) was also investigated in

HORCs. Glutamate is a highly abundant excitatory neurotransmitter (Schousboe 1981), extracellular levels of which have to be very tightly regulated to prevent neurotoxicity. In the retina, physiological glutamate concentrations are maintained through Na^+-dependent excitatory amino-acid transporters (EAATs), specifically glutamate–aspartate transporter (GLAST or EAAT1), glutamate transporter (GLT-1 or EAAT2), excitatory amino acid carrier 1 (EAAC1 or EAAT3) and excitatory amino acid transporter 4 and 5 (Danbolt 2001, Grewer & Rauen 2005, Marcaggi & Attwell 2004). It has been demonstrated on rat and human retinas that GLAST is expressed by ganglion cells and the glia, (Otori *et al.* 1994), as well as horizontal, bipolar, amacrine and photoreceptor cells (Kugler & Beyer 2003). In the rat retina, co-localization of glutamine synthase, an enzyme that convers glutamine to glutamate, and GLAST was also demonstrated on Müller cells, astrocytes, and the RPE (Derouiche & Rauen 1995). Whereas, the immunoreactivity of GLT-1 in the retina of rats and monkeys was found to be localized to cone photoreceptors and bipolar cells (Rauen & Kanner 1994). Retinal cells take up glutamate from extracellular space and convert it to glutamine by glutamine synthase. Glutamine is then transported into the neuronal pre-synaptic terminal via the extracellular space, where it is converted by glutaminase to glutamate (Marcaggi & Attwell 2004, Pow & Crook 1996). The results presented in this chapter showed no change in expression of *GLAST* mRNA indicating that TSA had no effect on glutamate metabolism in HORCs. The protective role of HDACIs against glutamate excitotoxicity in the brain, optic nerve and cultured neurons has been investigated in other *in vitro* studies. It has been

demonstrated that selective inhibition of HDAC 2 and 3 via AH51, AH61 and AH62 HDACIs prevented neuronal death against DL-threo-β-benzyloxyaspartate (DL-TBOA)-induced glutamate exitotoxic insult in cultured organotypic cerebral slices (Durham 2012). It has also been shown that non-selective HDAC inhibition using SAHA and MS-275 reduced accumulation of glutamate and up-regulated the expression of glutamate transporter GLT-1 on astrocytes *in vitro* using the optic nerve ischaemia model (Baltan *et al.* 2011). Moreover, the neuroprotective effect of non-selective HDACIs against glutamate excitotoxicity has also been shown using brain neuron cultures from rats, where both VPA and SB caused the up-regulation of pro-survival and anti-apoptotic genes, including heat shock protein-70 (*HSP70*) (Marinova *et al.* 2009) and *Bcl-2* (Leng *et al.* 2010).

Besides our main aim to evaluate the effect of TSA on RGC associated gene expression, the expression of other retinal cell markers was also measured to establish whether or not HDACIs are selective towards neuronal cells. Surprisingly, there was no change found in expression of recoverin (*RCVRN*), as a marker for photoreceptors (Dizhoor *et al.* 1991), calbindin (*CALB*), as a marker for horizontal cells (Nakhai *et al.* 2007), and glutamine synthetase (*Glul*), as a marker for Müller cells and astrocytes (Anlauf & Derouiche 2013, Riepe & Norenburg 1977). Although there was an increase in expression of choline acetyltransferase (*ChAT*) mRNA, a marker for cholinergic amacrine cells (Haverkamp & Wassle 2000), in HORCs treated with 1µM of TSA, the effect was not dose dependent. These results suggest that HDAC inhibition is preferential to survival of retinal ganglion cell. This suggestion is supported by

the study where the treatment of adult hippocampal neural progenitors with VPA promoted neuronal and inhibited glial cell differentiations through the induction of a neurogenic basic helix-loop-helix transcription factor, NeuroD (Hsieh *et al.* 2004). Moreover, it has also been shown that TSA treatment inhibited the proliferation of human RPE cells *in vitro* via down-regulation of TGF-β/Akt, MAPK, ERK1/2 and Notch signalling pathways (Xiao *et al.* 2014), and the development of mouse photoreceptors though inhibition of the expression of pro-rod transcription factors Otx2, Nrl, and Crx (Chen & Cepko 2007). However, there are several reports demonstrating a protective effect of HDACIs on photoreceptors. In a mouse model of retinitis pigmentosa, TSA treatment enhanced the photoreceptor cell survival and prevented photoreceptor degeneration by suppressing poly(ADP-ribose) polymerase (PARP) activity (Paquet-Durand *et al.* 2007, Sancho-Pelluz *et al.* 2010, Sancho-Pelluz & Paquet-Durand 2012).

The results of the current study also showed that there was a significant up-regulation of *HSP70* (*HSPA1B*) mRNA expression in HORCs under all culture conditions compared to that in t=0, indicating that activation of HSP expression is a potential mechanisms involved in regulation of cell survival in a long-term HORCs. It was also noticed that the treatment of HORCs with 10μM TSA exerted a tendency to up-regulate the expression of *HSP70* mRNA greater than in control cultures when both treatment conditions were compared to that in t=0 retinal explants. However, there was no statistically significant difference in expression of HSP70 mRNAs between controls and HORCs with 10μM TSA. HSPs is a superfamily of highly conserved proteins that are also known

as molecular chaperones (Hartl 1996). HSPs are involved in regulation of cellular homeostasis and promotion of cell survival by facilitating protein folding and degradation of abnormally folded proteins (Hartl 1996, Takayama *et al.* 2003). It is known that an increase in expression of HSPs is observed during normal cellular growth, and as a response of cells and tissues to acute and chronic stress conditions (Knowlton *et al.* 1998, Lindquist & Craig 1988, Nagashima *et al.* 2011, Tezel *et al.* 2000). A role of HSPs in glaucoma was demonstrated via a significant greater up-regulation of these proteins in human retinal explants from patients with known glaucoma when compared to that in controls (Tezel *et al.* 2000). Interestingly, it has been also reported that the central nervous system and motorneurons in culture have a tendency to have a delayed and selective heat shock response when compared with glial cells, suggesting these findings might be a potential explanation for increase susceptibility of neurons to neurodegenerative insults (Batulan *et al.* 2003, Marcuccilli *et al.* 1996, Tagawa *et al.* 2007). HSP70 is a part of the large HSP70 family. It was reported that HSP70 is specifically expressed in RGCs of the normal human retina (Ma 2013) and was shown to play a crucial role in RGC survival in the zebra fish retina after acute optic nerve injury (Nagashima *et al.* 2011). A protective effect of HDAC inhibition on RGC survival via overexpression of HSP70 was demonstrated *in vivo* using a rat ischemia/reperfusion model (Zhang *et al.* 2012). The neuroprotective effect was also associated with hyperacetylation of histone H3, reduced caspase 3 activity, upregulation of apoptotic-protease-activating factor-1 (apaf-1) and release of cytochrome C.

The present work demonstrates that HDAC inhibitor TSA can protect against loss of total RNA in cultured human retina, as well as possess a potential to attenuate specifically expression of RGC marker genes. There was no effect on RGC survival in 1 week HORCs. Therefore, it can be concluded that TSA can prevent RGC gene down-regulation in human retinal explants. However, TSA does not protect RGCs from apoptosis in a long-term HORC at concentrations tested.

CHAPTER 6

GENERAL SUMMARY AND DISCUSSION

Glaucoma is a complex disease where a combination of factors, including genetic predisposition (Ali *et al.* 2009, Copin *et al.* 2002, Hollander *et al.* 2006, Wolfs *et al.* 1998), vascular dysregulation (Broadway & Drance 1998, Drance *et al.* 2001, Leske 2009, Leske *et al.* 2002) and age (Dielemans *et al.* 1994, Heijl *et al.* 2013, Leske *et al.* 1994), stimulates a multitude of molecular pathways, which eventually result in an irreversible loss of RGCs and blindness. On the cellular level, glaucomatous RGC death involves retrograde RGC axon degeneration associated with the loss of the neighbouring cells. Human organotypic retinal cultures have been established as an *in vitro* model for investigation of mechanisms involved in RGC degeneration in glaucoma (Niyadurupola *et al.* 2013, Niyadurupola *et al.* 2011), and for development of new neuroprotective strategies. The preparation of the HORCs involve the transection of the optic nerve and retinal tissue dissection, followed by culture in controlled environment which resembles degenerative changes in the retinal tissue *in vivo*. Up to now, the fate of RGC beyond 96 hours in culture, the longest culture period previously investigated by Niyadurupola and colleagues (2011), remained unknown. The present study aimed to determine whether HORCs could be maintained *ex vivo* in culture for up to 4 weeks. In order to evaluate neurodegenerative changes in HORCs, several RGC markers were used. The first indicators used for investigation of the RGC fate were NeuN and TUNEL immunolabelling. In general, it was shown that over the first two

weeks in culture, there was a decrease in the number of NeuN-immunolabelled RGCs associated with an increase in the proportion of TUNEL-positive RGC nuclei. Since both markers reached a base line level at weeks 3 and 4 in culture, it can be concluded that first two weeks in culture is the most appropriate period for long-term experiments using HORCs. The expression of RGC-specific gene *Thy1* was also measured. A significant down-regulation in expression of *Thy1* mRNA in a first week of culture was observed, as was expected from previous work by Niyadurupola and colleagues (2011). A possible association in changes between RGC-specific markers expression, such as NeuN-immunolabelling and Thy1 mRNA, and TUNEL, as a marker for apoptosis, is presented in figure 49.

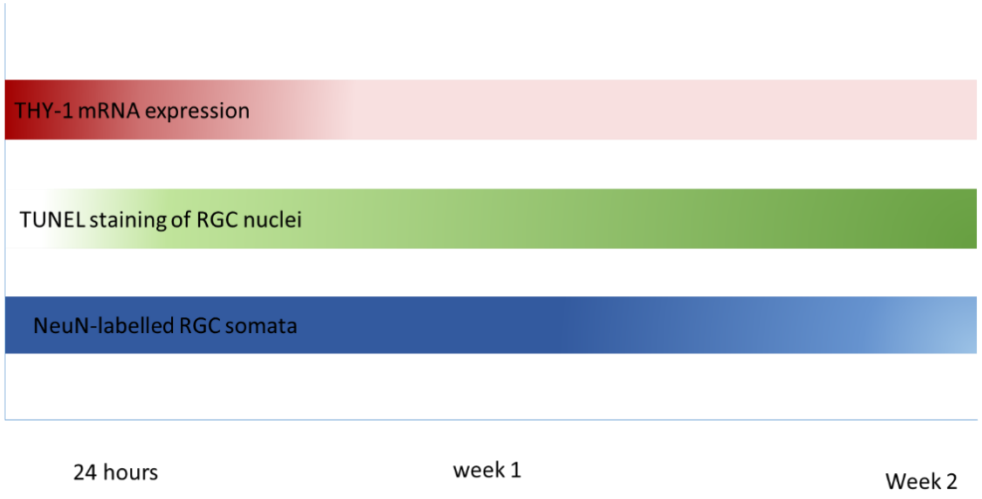


Figure 49: Schematic representation of changes observed in expression of RGC markers and TUNEL-immunolabelling in HORCs over 2 weeks in culture.

The current study also aimed to compare different culture conditions in order to establish the optimum medium to prolong RGC survival in HORCs. In contrast to DMEM/HamF12, Neurobasal medium was the most detrimental to

RGC survival in long-term HORCs, with the steepest increase in a number of TUNEL-positive RGCs at week 1 in culture. The effect of serum supplementation on the RGC fate in a long-term HORC was also estimated. There was very little difference in survival of RGCs between cultures with DMEM/HamF12 either with or without serum. These findings suggest that SF DMEM/HamF12 is the condition of choice for long-term experiments using HORCs. However, in order to detect a neuroprotective effect that would prevent NeuN-labelled RGC loss, as well as anti-apoptotic action, an experimental period of 2 weeks would be the most appropriate for HORCs with SF DMEM/HamF12. Whereas HORCs with Neurobasal medium would require only a period of 1 week to detect a significant level of RGC death.

In addition, in order to extend the survival of RGCs in HORCs as a long-term human model of RGC degeneration, several MSC-derived growth factors were tested. It was found that both VEGF and PDGF-AA significantly reduced the proportion of apoptotic RGCs after 1 week in culture, indicating a strong neurotrophic effect of these factors to RGCs in the human retina. There is a possibility that in combination VEGF and PDGF-AA would exert an even more prominent protective effect, but that suggestion was not investigated in the current study. However, it can be followed from the above results that supplementation with VEGF and PDGD-AA, on its own or in combination, would prolong RGC survival in a long-term HORC. It would also be useful to assess an effect of these neurotrophic factors on the RGC survival in other models of RGC degeneration in humans, for example, oxidative stress and exitotoxicity. In general, supplementation with growth factors has been

extensively used as a strategy to stimulate cell proliferation, differentiation or to express specific cell functions *in vitro* prior to an insult. Traditionally, growth factors were added to cell and tissue cultures via supplementation with serum. However, due to the need for chemically defined and consistent culture conditions, further investigations led to identification of specific growth factors that enhanced neuronal cell survival. Several studies both *in vivo* and *in vitro* have shown that numerous growth factors, including basic fibroblast growth factor (FGF2), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), pigment epithelium–derived factor (PEDF), and vascular endothelial growth factor (VEGF), were able to increased RGC survival and axon regeneration (Blanco *et al.* 2000, Klocker *et al.* 1997, Klocker *et al.* 2000, Mey & Thanos 1993, Nishijima *et al.* 2007, Pang *et al.* 2007, Webber *et al.* 2005). It has also been demonstrated that a combination of growth factors exerts an additive effect on the RGC survival *in vivo* (Watanabe *et al.* 2003). Moreover, it has been shown that supplementing growth factors with additional factors, for example forskolin or hormones, further improved RGC survival and stimulated their regeneration *in vivo* (Watanabe *et al.* 2003) and *in vitro* (Meyer-Franke *et al.* 1995, Toops *et al.* 2012). Importantly, the present study was specifically interested in growth factors known to be secreted by bmMSC (Johnson *et al.* 2014). The relevance of VEGF and PDGF neurotrophic effects to RGCs is not only in their potential to be used as culture medium supplements, but also in their role as serious candidates for development of future therapeutic strategies against glaucoma. Moreover, the findings of the current study also support

concerns about increasing interest in combining anti-VEGF/PDGF treatments for ocular neovascularization and proliferative eye diseases either via blockage of growth factors expression or binding to their receptors (Liegl *et al.* 2014, Mendel *et al.* 2003, Takahashi *et al.* 2009).

Down-regulation of specific for RGCs gene expression is a well-documented early event in a process of RGC degeneration representing the commitment of these cells to death via initiation of apoptotic cell death pathways (Pelzel *et al.* 2012, Schlamp *et al.* 2001, Wang *et al.* 2010a, Weishaupt *et al.* 2005, Yang *et al.* 2007). It is also know that one of the major mechanisms involved in the control of gene expression is a reversible regulation of chromatin organization facilitated via action of two enzymes with apposite functions, HAT and HDAC. Activation of HDACs is implicated in deacetylation of histones and non-histone proteins leading to chromatin condensation and subsequent alterations in gene expression required for healthy cells to function. It was shown *in vivo*, using a mouse model of acute ON crush, that a significant reduction in the RGC loss was associated with overexpression of acetylated histone H4, as well as an up-regulation of the RGC maker Fem1cR3 gene after pretreatment with histone deacetylase inhibitor TSA. In current work, the effect of inhibition of retinal HDAC activity using TSA was investigated in HORCs. It was found that TSA exerted a selective protective effect against RGC-related mRNA loss, however there was no protection observed against apoptotic RGC death after 1 week in culture. It possible to suggest that for RGC survival over time, the support of other cells is require, evidence of that support was not found in the presented work.

Some would argue that the speed of the changes observed in HORCs might be more relevant to pathophysiological changes found in acute glaucoma following, for example, an acute elevation in IOP rather than chronic glaucoma. Clinically, acute glaucoma is characterised by an acute insult to the optic nerve with a rapid onset of the optic nerve cupping, whereas in chronic glaucoma the loss of the RGCs is slow and can take months to years to develop. Others, however, might point out that human tissue culture is more likely to represent the advanced stages of chronic glaucoma, because of the complete transection of the optic nerve with no possibility of the reversal of the RGC fate. HORC is a universal model allowing analysis of both the acute changes in gene expression before the loss of RGCs becomes detectable, most likely to be observed in patients with acute glaucoma, as well as quantifiable loss of the neurons over time relevant to chronic glaucoma. Knowing that the retinal explant is a viable tissue over the extended period of time facilitates a flexible approach to the timing of the tested interventions because morphological changes that are seen after days to weeks in culture can potentially relate to months and years of the glaucomatous neurodegeneration.

HORC is a valuable model for investigation of mechanisms involved in human RGC degeneration and can be utilised as an important system for neuroprotective studies in the future.

REFERENCES

Adamis AP, Shima DT, Yeo KT, Yeo TK, Brown LF, Berse B*, et al.* (1993) Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. *Biochem Biophys Res Commun* 193: 631-638.

AGIS. (2000) The Advanced Glaucoma Intervention Study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration. *Am J Ophthalmol* 130: 429-440.

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: 1247-1258.

Ali M, McKibbin M, Booth A, Parry DA, Jain P, Riazuddin SA*, et al.* (2009) Null mutations in LTBP2 cause primary congenital glaucoma. *Am J Hum Genet* 84: 664-671.

Allen NJ & Barres BA. (2009) Neuroscience: Glia - more than just brain glue. *Nature* 457: 675-677.

Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W*, et al.* (2001) Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. *Mol Cell Neurosci* 18: 270-282.

Ames A. (1992) Energy requirements of CNS cells as related to their function and to their vulnerability to ischemia: a commentary based on studies on retina. *Can J Physiol Pharmacol* 70 Suppl: S158-164.

Ames A, Li YY, Heher EC & Kimble CR. (1992) Energy metabolism of rabbit retina as related to function: high cost of Na+ transport. *J Neurosci* 12: 840-853.

Anderson DR. (1969) Ultrastructure of Human and Monkey Lamina Cribrosa and Optic Nerve Head. *Arch Ophthalmol* 82: 800-814.

Anderson DR. (1999) Introductory comments on blood flow autoregulation in the optic nerve head and vascular risk factors in glaucoma. *Surv Ophthalmol* 43 Suppl 1: S5-9.

Anderson DR & Hendrickson A. (1974) Effect of intraocular pressure on rapid axoplasmic transport in monkey optic nerve. *Invest Ophthalmol* 13: 771-783.

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. *Nat Genet* 30: 81-85.

Anlauf E & Derouiche A. (2013) Glutamine synthetase as an astrocytic marker: its cell type and vesicle localization. *Front Endocrinol (Lausanne)* 4: 144.

Aoun P, Simpkins JW & Agarwal N. (2003) Role of PPAR-gamma ligands in neuroprotection against glutamate-induced cytotoxicity in retinal ganglion cells. *Invest Ophthalmol Vis Sci* 44: 2999-3004.

Araki CM & Hamassaki-Britto DE. (2000) Calretinin co-localizes with the NMDA receptor subunit NR1 in cholinergic amacrine cells of the rat retina. *Brain Res* 869: 220-224.

Arents G, Burlingame RW, Wang BC, Love WE & Moudrianakis EN. (1991) The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. *Proc Natl Acad Sci U S A* 88: 10148-10152.

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

Ashkenazi A & Dixit VM. (1998) Death receptors: signaling and modulation. *Science* 281: 1305-1308.

Attwell D, Barbour B & Szatkowski M. (1993) Nonvesicular release of neurotransmitter. *Neuron* 11: 401-407.

Azari MF, Galle A, Lopes EC, Kurek J & Cheema SS. (2001) Leukemia inhibitory factor by systemic administration rescues spinal motor neurons in the SOD1 G93A murine model of familial amyotrophic lateral sclerosis. *Brain Res* 922: 144-147.

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. *Mol Brain* 6: 34.

Baimbridge KG, Celio MR & Rogers JH. (1992) Calcium-binding proteins in the nervous system. *Trends Neurosci* 15: 303-308.

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. *Clin Ophthalmol* 5: 553-556.

Balasubramaniam B, Carter DA, Mayer EJ & Dick AD. (2009) Microglia derived IL-6 suppresses neurosphere generation from adult human retinal cell suspensions. *Exp Eye Res* 89: 757-766.

Balazsi AG, Rootman J, Drance SM, Schulzer M & Douglas GR. (1984) The effect of age on the nerve fiber population of the human optic nerve. *Am J Ophthalmol* 97: 760-766.

Bali P, Pranpat M, Bradner J, Balasis M, Fiskus W, Guo F*, et al.* (2005) Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *J Biol Chem* 280: 26729-26734.

Ball SG, Shuttleworth A & Kielty CM. (2012) Inhibition of platelet-derived growth factor receptor signaling regulates Oct4 and Nanog expression, cell shape, and mesenchymal stem cell potency. *Stem Cells* 30: 548-560.

Baltan S, Murphy SP, Danilov CA, Bachleda A & Morrison RS. (2011) Histone deacetylase inhibitors preserve white matter structure and function during ischemia by conserving ATP and reducing excitotoxicity. *J Neurosci* 31: 3990-3999.

Bao X, Wei J, Feng M, Lu S, Li G, Dou W*, et al.* (2011) Transplantation of human bone marrow-derived mesenchymal stem cells promotes behavioral recovery and endogenous neurogenesis after cerebral ischemia in rats. *Brain Res* 1367: 103-113.

Barker FM, 2nd, Snodderly DM, Johnson EJ, Schalch W, Koepcke W, Gerss J & Neuringer M. (2011) Nutritional manipulation of primate retinas, V: effects of lutein, zeaxanthin, and n-3 fatty acids on retinal sensitivity to blue-light-induced damage. *Invest Ophthalmol Vis Sci* 52: 3934-3942.

Barnstable CJ & Drager UC. (1984) Thy-1 antigen: a ganglion cell specific marker in rodent retina. *Neuroscience* 11: 847-855.

Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN*, et al.* (1997) Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. *Stroke* 28: 1233-1244.

Barres BA, Silverstein BE, Corey DP & Chun LL. (1988) Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron* 1: 791-803.

Batulan Z, Shinder GA, Minotti S, He BP, Doroudchi MM, Nalbantoglu J*, et al.* (2003) High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. *J Neurosci* 23: 5789-5798.

Bauer S & Patterson PH. (2006) Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. *J Neurosci* 26: 12089-12099.

Beckermann BM, Kallifatidis G, Groth A, Frommhold D, Apel A, Mattern J*, et al.* (2008) VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer* 99: 622-631.

Beier M, Franke A, Paunel-Gorgulu AN, Scheerer N & Dunker N. (2006) Transforming growth factor beta mediates apoptosis in the ganglion cell layer during all programmed cell death periods of the developing murine retina. *Neurosci Res* 56: 193-203.

Benjamin LE, Hemo I & Keshet E. (1998) A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 125: 1591-1598.

Bergendi L, Benes L, Durackova Z & Ferencik M. (1999) Chemistry, physiology and pathology of free radicals. *Life Sciences* 65: 1865-1874.

Bergers G, Song S, Meyer-Morse N, Bergsland E & Hanahan D. (2003) Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 111: 1287-1295.

Berkelaar M, Clarke DB, Wang YC, Bray GM & Aguayo AJ. (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci* 14: 4368-4374.

Bernstein PS, Khachik F, Carvalho LS, Muir GJ, Zhao DY & Katz NB. (2001) Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. *Exp Eye Res* 72: 215-223.

Birich TV, Birich TA, Marchenko LN, Remizonova DB & Fedulov AS. (1986) Perekisnoe okislenie lipidov v krovi bol'nykh pervichnoi glaukomoi (Lipid peroxidation in the blood of primary glaucoma patients). *Vestn Oftalmol* 102: 13-15.

Biswas SK, Zhao Y, Nagalingam A, Gardner TW & Sandirasegarane L. (2008) PDGF- and insulin/IGF-1-specific distinct modes of class IA PI 3-kinase activation in normal rat retinas and RGC-5 retinal ganglion cells. *Invest Ophthalmol Vis Sci* 49: 3687-3698.

Blanco RE, Lopez-Roca A, Soto J & Blagburn JM. (2000) Basic fibroblast growth factor applied to the optic nerve after injury increases long-term cell survival in the frog retina. *J Comp Neurol* 423: 646-658.

Blesch A, Uy HS, Grill RJ, Cheng JG, Patterson PH & Tuszynski MH. (1999) Leukemia inhibitory factor augments neurotrophin expression and corticospinal axon growth after adult CNS injury. *J Neurosci* 19: 3556-3566.

Bogaert E, Van Damme P, Poesen K, Dhondt J, Hersmus N, Kiraly D*, et al.* (2010) VEGF protects motor neurons against excitotoxicity by upregulation of GluR2. *Neurobiol Aging* 31: 2185-2191.

Bohannon LK, Owens SD, Walker NJ, Carrade DD, Galuppo LD & Borjesson DL. (2013) The effects of therapeutic concentrations of gentamicin, amikacin and hyaluronic acid on cultured bone marrow-derived equine mesenchymal stem cells. *Equine Vet J* 45: 732-736.

Bonde C, Sarup A, Schousboe A, Gegelashvili G, Noraberg J & Zimmer J. (2003) GDNF pre-treatment aggravates neuronal cell loss in oxygen-glucose deprived hippocampal slice cultures: a possible effect of glutamate transporter up-regulation. *Neurochem Int* 43: 381-388.

Bonomi L, Marchini G, Marraffa M, Bernardi P, Morbio R & Varotto A. (2000) Vascular risk factors for primary open angle glaucoma: the Egna-Neumarkt Study. *Ophthalmology* 107: 1287-1293.

Bose P, Dai Y & Grant S. (2014) Histone deacetylase inhibitor (HDACI) mechanisms of action: Emerging insights. *Pharmacol Ther* 143: 323-336.

Bozanic D, Bocina I & Saraga-Babic M. (2006) Involvement of cytoskeletal proteins and growth factor receptors during development of the human eye. *Anat Embryol (Berl)* 211: 367-377.

Breier G, Albrecht U, Sterrer S & Risau W. (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114: 521-532.

Brewer GJ, Torricelli JR, Evege EK & Price PJ. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35: 567-576.

Broadway DC & Drance SM. (1998) Glaucoma and vasospasm. *Br J Ophthalmol* 82: 862-870.

Broide RS, Redwine JM, Aftahi N, Young W, Bloom FE & Winrow CJ. (2007) Distribution of histone deacetylases 1-11 in the rat brain. *J Mol Neurosci* 31: 47-58.

Brooks DE. (1990) Glaucoma in the dog and cat. *Vet Clin North Am Small Anim Pract* 20: 775-797.

Brooks DE, Garcia GA, Dreyer EB, Zurakowski D & Franco-Bourland RE. (1997) Vitreous body glutamate concentration in dogs with glaucoma. *Am J Vet Res* 58: 864-867.

Brown DM, Michels M, Kaiser PK, Heier JS, Sy JP, Ianchulev T & Group AS. (2009) Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: Two-year results of the ANCHOR study. *Ophthalmology* 116: 57-65 e55.

Brown PK & Wald G. (1964) Visual Pigments in Single Rods and Cones of the Human Retina. Direct Measurements Reveal Mechanisms of Human Night and Color Vision. *Science* 144: 45-52.

Buchanan CD, Mahesh VB & Brann DW. (2000) Estrogen-astrocyte-luteinizing hormone-releasing hormone signaling: a role for transforming growth factor-beta(1). *Biol Reprod* 62: 1710-1721.

Buckingham BP, Inman DM, Lambert W, Oglesby E, Calkins DJ, Steele MR*, et al.* (2008) Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J Neurosci* 28: 2735-2744.

Bull ND, Johnson TV, Welsapar G, DeKorver NW, Tomarev SI & Martin KR. (2011) Use of an adult rat retinal explant model for screening of potential retinal ganglion cell neuroprotective therapies. *Invest Ophthalmol Vis Sci* 52: 3309-3320.

Burgi S, Samardzija M & Grimm C. (2009) Endogenous leukemia inhibitory factor protects photoreceptor cells against light-induced degeneration. *Mol Vis* 15: 1631-1637.

Burgoyne CF, Downs JC, Bellezza AJ & Hart RT. (2004) Three-dimensional reconstruction of normal and early glaucoma monkey optic nerve head connective tissues. *Invest Ophthalmol Vis Sci* 45: 4388-4399.

Burgoyne CF, Downs JC, Bellezza AJ, Suh JKF & Hart RT. (2005) The optic nerve head as a biomechanical structure: a new paradigm for understanding the role of IOP-related stress and strain in the pathophysiology of glaucomatous optic nerve head damage. *Prog Retin Eye Res* 24: 39-73.

Cadenas E & Sies H. (1998) The lag phase. *Free Radic Res* 28: 601-609.

Caffe AR, Ahuja P, Holmqvist B, Azadi S, Forsell J, Holmqvist I*, et al.* (2001) Mouse retina explants after long-term culture in serum free medium. *J Chem Neuroanat* 22: 263-273.

Cairns BR. (2009) The logic of chromatin architecture and remodelling at promoters. *Nature* 461: 193-198.

Campochiaro PA & Conway BP. (1991) Aminoglycoside toxicity--a survey of retinal specialists. Implications for ocular use. *Arch Ophthalmol* 109: 946-950.

Campochiaro PA, Hackett SF, Vinores SA, Freund J, Csaky C, LaRochelle W*, et al.* (1994) Platelet-derived growth factor is an autocrine growth stimulator in retinal pigmented epithelial cells. *J Cell Sci* 107 (Pt 9): 2459-2469.

Carr AJ, Vugler A, Lawrence J, Chen LL, Ahmado A, Chen FK*, et al.* (2009) Molecular characterization and functional analysis of phagocytosis by human embryonic

stem cell-derived RPE cells using a novel human retinal assay. *Mol Vis* 15: 283-295.

Carter DA & Dick AD. (2003) Lipopolysaccharide/interferon-gamma and not transforming growth factor beta inhibits retinal microglial migration from retinal explant. *Br J Ophthalmol* 87: 481-487.

Chan AS, Saraswathy S, Rehak M, Ueki M & Rao NA. (2012) Neuroglobin protection in retinal ischemia. *Invest Ophthalmol Vis Sci* 53: 704-711.

Chang B, Smith RS, Hawes NL, Anderson MG, Zabaleta A, Savinova O*, et al.* (1999) Interacting loci cause severe iris atrophy and glaucoma in DBA/2J mice. *Nat Genet* 21: 405-409.

Chang ML, Wu CH, Jiang-Shieh YF, Shieh JY & Wen CY. (2007) Reactive changes of retinal astrocytes and Muller glial cells in kainate-induced neuroexcitotoxicity. *J Anat* 210: 54-65.

Cheema SS, Richards L, Murphy M & Bartlett PF. (1994) Leukemia inhibitory factor prevents the death of axotomised sensory neurons in the dorsal root ganglia of the neonatal rat. *J Neurosci Res* 37: 213-218.

Chen B & Cepko CL. (2007) Requirement of histone deacetylase activity for the expression of critical photoreceptor genes. *BMC Dev Biol* 7: 78-90.

Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L & Stewart CL. (2000) Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology* 141: 4365-4372.

Chen PS, Peng GS, Li G, Yang S, Wu X, Wang CC*, et al.* (2006) Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol Psychiatry* 11: 1116-1125.

Chidlow G, Wood JP & Casson RJ. (2007) Pharmacological neuroprotection for glaucoma. *Drugs* 67: 725-759.

Choi HB, Gordon GRJ, Zhou N, Tai C, Rungta RL, Martinez J*, et al.* (2012) Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase. *Neuron* 75: 1094-1104.

Choi JH, Kwon HJ, Yoon BI, Kim JH, Han SU, Joo HJ & Kim DY. (2001) Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn J Cancer Res* 92: 1300-1304.

Chollangi S, Wang J, Martin A, Quinn J & Ash JD. (2009) Preconditioning-induced protection from oxidative injury is mediated by leukemia inhibitory factor receptor (LIFR) and its ligands in the retina. *Neurobiol Dis* 34: 535-544.

Chong V. (2012) Biological, preclinical and clinical characteristics of inhibitors of vascular endothelial growth factors. *Ophthalmologica* 227 Suppl 1: 2-10.

Choritz L, Machert M & Thieme H. (2012) Correlation of endothelin-1 concentration in aqueous humor with intraocular pressure in primary open angle and pseudoexfoliation glaucoma. *Invest Ophthalmol Vis Sci* 53: 7336-7342.

Christensen DP, Dahllof M, Lundh M, Rasmussen DN, Nielsen MD, Billestrup N*, et al.* (2011) Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus. *Mol Med* 17: 378-390.

Chu Y, Hughes S & Chan-Ling T. (2001) Differentiation and migration of astrocyte precursor cells and astrocytes in human fetal retina: relevance to optic nerve coloboma. *FASEB J* 15: 2013-2015.

Chu Y, Humphrey MF & Constable IJ. (1993) Horizontal cells of the normal and dystrophic rat retina: a wholemount study using immunolabelling for the 28-kDa calcium-binding protein. *Exp Eye Res* 57: 141-148.

Ciarlo E, Savva A & Roger T. (2013) Epigenetics in sepsis: targeting histone deacetylases. *Int J Antimicrob Agents* 42 Suppl: S8-12.

Cohen SY, Oubraham H, Uzzan J, Dubois L & Tadayoni R. (2012) Causes of unsuccessful ranibizumab treatment in exudative age-related macular degeneration in clinical settings. *Retina* 32: 1480-1485.

Collaborative. (1998) The effectiveness of intraocular pressure reduction in the treatment of normal-tension glaucoma. Collaborative Normal Tension Glaucoma Study Group. *Am J Ophthalmol* 126: 498-505.

Congdon N, Wang F & Tielsch JM. (1992) Issues in the Epidemiology and Population-Based Screening of Primary Angle-Closure Glaucoma. *Surv Ophthalmol* 36: 411-423.

Congdon NG, Qi YL, Quigley H, Hung PT, Wang TH, Ho TC & Tielsch JM. (1997) Biometry and primary angle-closure glaucoma among Chinese, white, and black populations. *Ophthalmology* 104: 1489-1495.

Copin B, Brezin AP, Valtot F, Dascotte JC, Bechetoille A & Garchon HJ. (2002) Apolipoprotein E-promoter single-nucleotide polymorphisms affect the phenotype of primary open-angle glaucoma and demonstrate interaction with the myocilin gene. *Am J Hum Genet* 70: 1575-1581.

Cressey D. (2009) Neuroscientists claim growing pains. *Nature* 459: 19-20.

Cui XS, Xu YN, Shen XH, Zhang LQ, Zhang JB & Kim NH. (2011) Trichostatin A modulates apoptotic-related gene expression and improves embryo viability in cloned bovine embryos. *Cell Reprogram* 13: 179-189.

Curcio CA & Allen KA. (1990) Topography of ganglion cells in human retina. *The Journal of comparative neurology* 300: 5-25.

Dacey DM, Liao HW, Peterson BB, Robinson FR, Smith VC, Pokorny J*, et al.* (2005) Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature* 433: 749-754.

Dai C, Khaw PT, Yin ZQ, Li D, Raisman G & Li Y. (2012) Structural basis of glaucoma: the fortified astrocytes of the optic nerve head are the target of raised intraocular pressure. *Glia* 60: 13-28.

Dai Y, Guzman ML, Chen S, Wang L, Yeung SK, Pei XY*, et al.* (2010) The NF (Nuclear factor)-kappaB inhibitor parthenolide interacts with histone deacetylase inhibitors to induce MKK7/JNK1-dependent apoptosis in human acute myeloid leukaemia cells. *Br J Haematol* 151: 70-83.

Dalton R. (2001) Private investigations. *Nature* 411: 129-130.

Danbolt NC. (2001) Glutamate uptake. *Prog Neurobiol* 65: 1-105.

Danial NN & Korsmeyer SJ. (2004) Cell death: critical control points. *Cell* 116: 205-219.

Davies ER, Haitchi HM, Thatcher TH, Sime PJ, Kottmann RM, Ganesan A*, et al.* (2012) Spiruchostatin A inhibits proliferation and differentiation of fibroblasts from patients with pulmonary fibrosis. *Am J Respir Cell Mol Biol* 46: 687-694.

Davis ID, Kiers L, MacGregor L, Quinn M, Arezzo J, Green M*, et al.* (2005) A randomized, double-blinded, placebo-controlled phase II trial of recombinant human leukemia inhibitory factor (rhuLIF, emfilermin, AM424) to prevent chemotherapy-induced peripheral neuropathy. *Clin Cancer Res* 11: 1890-1898.

De Bock F, Dornand J & Rondouin G. (1996) Release of TNF alpha in the rat hippocampus following epileptic seizures and excitotoxic neuronal damage. *Neuroreport* 7: 1125-1129.

De Kater AW, Smyth JR, Jr., Rosenquist RC & Epstein DL. (1986) The Slate turkey: a model for secondary angle closure glaucoma. *Invest Ophthalmol Vis Sci* 27: 1751-1754.

Del Rio P, Irmler M, Arango-Gonzalez B, Favor J, Bobe C, Bartsch U*, et al.* (2011) GDNF-induced osteopontin from Muller glial cells promotes photoreceptor survival in the Pde6brd1 mouse model of retinal degeneration. *Glia* 59: 821-832.

Deroanne CF, Bonjean K, Servotte S, Devy L, Colige A, Clausse N*, et al.* (2002) Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. *Oncogene* 21: 427-436.

Derouiche A & Rauen T. (1995) Coincidence of L-glutamate/L-aspartate transporter (GLAST) and glutamine synthetase (GS) immunoreactions in retinal glia: evidence for coupling of GLAST and GS in transmitter clearance. *J Neurosci Res* 42: 131-143.

Diaz F, Villena A, Moreno M, Vidal L, Parrado C, Garcia-Campos J & de Vargas IP. (2005) Effects of a non-selective beta-blocker on adult rat anterograde axonal transport and retinal ganglion layer after increased intraocular pressure. *Histol Histopathol* 20: 1077-1084.

Dielemans I, Vingerling JR, Wolfs RC, Hofman A, Grobbee DE & de Jong PT. (1994) The prevalence of primary open-angle glaucoma in a population-based study in The Netherlands. The Rotterdam Study. *Ophthalmology* 101: 1851-1855.

Dizhoor AM, Ray S, Kumar S, Niemi G, Spencer M, Brolley D*, et al.* (1991) Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. *Science* 251: 915-918.

Dong CJ, Guo Y, Agey P, Wheeler L & Hare WA. (2008) Alpha2 adrenergic modulation of NMDA receptor function as a major mechanism of RGC protection in experimental glaucoma and retinal excitotoxicity. *Invest Ophthalmol Vis Sci* 49: 4515-4522.

Dong CJ, Guo Y, Wheeler L & Hare WA. (2007) Alpha2 adrenergic receptor-mediated modulation of cytosolic Ca++ signals at the inner plexiform layer of the rat retina. *Invest Ophthalmol Vis Sci* 48: 1410-1415.

Donovan SL & Dyer MA. (2006) Preparation and square wave electroporation of retinal explant cultures. *Nat Protoc* 1: 2710-2718.

Dowsing BJ, Hayes A, Bennett TM, Morrison WA & Messina A. (2000) Effects of LIF dose and laminin plus fibronectin on axotomized sciatic nerves. *Muscle Nerve* 23: 1356-1364.

Drance S, Anderson DR & Schulzer M. (2001) Risk factors for progression of visual field abnormalities in normal-tension glaucoma. Collaborative Normal-Tension Glaucoma Study Group. *Am J Ophthalmol* 131: 699-708.

Dredge BK & Jensen KB. (2011) NeuN/Rbfox3 nuclear and cytoplasmic isoforms differentially regulate alternative splicing and nonsense-mediated decay of Rbfox2. *PLoS One* 6: e21585.

Dreyer EB, Zurakowski D, Schumer RA, Podos SM & Lipton SA. (1996) Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. *Arch Ophthalmol* 114: 299-305.

Driessen CA, Winkens HJ, Hoffmann K, Kuhlmann LD, Janssen BP, Van Vugt AH*, et al.* (2000) Disruption of the 11-cis-retinol dehydrogenase gene leads to accumulation of cis-retinols and cis-retinyl esters. *Mol Cell Biol* 20: 4275-4287.

Duenas M, Luquin S, Chowen JA, Torres-Aleman I, Naftolin F & Garcia-Segura LM. (1994) Gonadal hormone regulation of insulin-like growth factor-I-like immunoreactivity in hypothalamic astroglia of developing and adult rats. *Neuroendocrinology* 59: 528-538.

Dun Y, Thangaraju M, Prasad P, Ganapathy V & Smith SB. (2007) Prevention of excitotoxicity in primary retinal ganglion cells by (+)-pentazocine, a sigma receptor-1 specific ligand. *Invest Ophthalmol Vis Sci* 48: 4785-4794.

Durham B. (2012) Novel histone deacetylase (HDAC) inhibitors with improved selectivity for HDAC2 and 3 protect against neural cell death. *Bioscience Horizons* 5.

Duval D, Reinhardt B, Kedinger C & Boeuf H. (2000) Role of suppressors of cytokine signaling (Socs) in leukemia inhibitory factor (LIF) -dependent embryonic stem cell survival. *FASEB J* 14: 1577-1584.

Edens RE, Dagtas S & Gilbert KM. (2006) Histone deacetylase inhibitors induce antigen specific anergy in lymphocytes: a comparative study. *Int Immunopharmacol* 6: 1673-1681.

Egawa-Tsuzuki T, Ohno M, Tanaka N, Takeuchi Y, Uramoto H, Faigle R*, et al.* (2004) The PDGF B-chain is involved in the ontogenic susceptibility of the developing rat brain to NMDA toxicity. *Exp Neurol* 186: 89-98.

Elmore S. (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35: 495-516.

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP*, et al.* (2004) Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB J* 18: 338-340.

Estrada R, Li N, Sarojini H, An J, Lee MJ & Wang E. (2009) Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. *J Cell Physiol* 219: 563-571.

Famiglietti EV, Stopa EG, McGookin ED, Song P, LeBlanc V & Streeten BW. (2003) Immunocytochemical localization of vascular endothelial growth factor in neurons and glial cells of human retina. *Brain Res* 969: 195-204.

Farinelli SE & Nicklas WJ. (1992) Glutamate metabolism in rat cortical astrocyte cultures. *J Neurochem* 58: 1905-1915.

Feigenspan A, Bormann J & Wassle H. (1993) Organotypic slice culture of the mammalian retina. *Vis Neurosci* 10: 203-217.

Fernandez-Bueno I, Fernandez-Sanchez L, Gayoso MJ, Garcia-Gutierrez MT, Pastor JC & Cuenca N. (2012) Time course modifications in organotypic culture of human neuroretina. *Exp Eye Res* 104: 26-38.

Fernandez-Bueno I, Pastor JC, Gayoso MJ, Alcalde I & Garcia MT. (2008) Muller and macrophage-like cell interactions in an organotypic culture of porcine neuroretina. *Mol Vis* 14: 2148-2156.

Ferrara N. (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280: C1358-1366.

Ferreira SM, Lerner SF, Brunzini R, Evelson PA & Llesuy SF. (2004) Oxidative stress markers in aqueous humor of glaucoma patients. *Am J Ophthalmol* 137: 62-69.

Ferrer-Martin RM, Martin-Oliva D, Sierra A, Carrasco MC, Martin-Estebane M, Calvente R*, et al.* (2014) Microglial cells in organotypic cultures of developing and adult mouse retina and their relationship with cell death. *Exp Eye Res* 121: 42-57.

Fingert JH, Heon E, Liebmann JM, Yamamoto T, Craig JE, Rait J*, et al.* (1999) Analysis of myocilin mutations in 1703 glaucoma patients from five different populations. *Hum Mol Genet* 8: 899-905.

Finnemann SC. (2003) Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *EMBO J* 22: 4143-4154.

Flores C, Salmaso N, Cain S, Rodaros D & Stewart J. (1999) Ovariectomy of adult rats leads to increased expression of astrocytic basic fibroblast growth factor in the ventral tegmental area and in dopaminergic projection regions of the entorhinal and prefrontal cortex. *J Neurosci* 19: 8665-8673.

Fonseca CP, Gama S, Saavedra A & Baltazar G. (2014) HO- or l-DOPA-injured dopaminergic neurons trigger the release of soluble mediators that up-regulate striatal GDNF through different signalling pathways. *Biochim Biophys Acta* 1842: 927-934.

Forrester JV, Dick AD, McMenamin PG & Roberts F. (2008) The Eye: Basic science in practice. *Edinburgh: Saunders*.

Foster PJ & Johnson GJ. (2001) Glaucoma in China: how big is the problem? *Br J Ophthalmol* 85: 1277-1282.

Foxton RH, Finkelstein A, Vijay S, Dahlmann-Noor A, Khaw PT, Morgan JE*, et al.* (2013) VEGF-A is necessary and sufficient for retinal neuroprotection in models of experimental glaucoma. *Am J Pathol* 182: 1379-1390.

Franke H & Illes P. (2014) Pathological potential of astroglial purinergic receptors. *Adv Neurobiol* 11: 213-256.

Fredriksson L, Ehnman M, Fieber C & Eriksson U. (2005) Structural requirements for activation of latent platelet-derived growth factor CC by tissue plasminogen activator. *J Biol Chem* 280: 26856-26862.

Fruttiger M, Karlsson L, Hall AC, Abramsson A, Calver AR, Bostrom H*, et al.* (1999) Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* 126: 457-467.

Fuentes-Prior P & Salvesen GS. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 384: 201-232.

Gaasterland D & Kupfer C. (1974) Experimental glaucoma in the rhesus monkey. *Invest Ophthalmol* 13: 455-457.

Galassi F, Sodi A, Ucci F, Renieri G, Pieri B & Baccini M. (2003) Ocular hemodynamics and glaucoma prognosis: a color Doppler imaging study. *Arch Ophthalmol* 121: 1711-1715.

Gao H, Qiao X, Cantor LB & WuDunn D. (2002) Up-regulation of brain-derived neurotrophic factor expression by brimonidine in rat retinal ganglion cells. *Arch Ophthalmol* 120: 797-803.

Garcia-Valenzuela E, Shareef S, Walsh J & Sharma SC. (1995) Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp Eye Res* 61: 33-44.

Ge XQ & Lin H. (2014) Noncoding RNAs in the regulation of DNA replication. *Trends Biochem Sci* 39: 341-343.

Gendron RL. (1999) A plasticity for blood vessel remodeling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Surv Ophthalmol* 44: 184-185.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V & Ferrara N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273: 30336-30343.

Gerometta R, Spiga MG, Borras T & Candia OA. (2010) Treatment of sheep steroid-induced ocular hypertension with a glucocorticoid-inducible MMP1 gene therapy virus. *Invest Ophthalmol Vis Sci* 51: 3042-3048.

Gherezghiher T, March WF, Nordquist RE & Koss MC. (1986) Laser-induced glaucoma in rabbits. *Exp Eye Res* 43: 885-894.

Gherghel D, Griffiths HR, Hilton EJ, Cunliffe IA & Hosking SL. (2005) Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. *Invest Ophthalmol Vis Sci* 46: 877-883.

Gitay-Goren H, Cohen T, Tessler S, Soker S, Gengrinovitch S, Rockwell P*, et al.* (1996) Selective binding of VEGF121 to one of the three vascular endothelial growth factor receptors of vascular endothelial cells. *J Biol Chem* 271: 5519-5523.

Gluzman-Poltorak Z, Cohen T, Herzog Y & Neufeld G. (2000) Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *J Biol Chem* 275: 18040-18045.

Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR & Group VISiONCT. (2004) Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 351: 2805-2816.

Graham SL & Drance SM. (1999) Nocturnal hypotension: role in glaucoma progression. *Surv Ophthalmol* 43 Suppl 1: S10-16.

Grant S & Dai Y. (2012) Histone deacetylase inhibitors and rational combination therapies. *Adv Cancer Res* 116: 199-237.

Green DR. (2000) Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 102: 1-4.

Green DR & Kroemer G. (2004) The pathophysiology of mitochondrial cell death. *Science* 305: 626-629.

Grewer C & Rauen T. (2005) Electrogenic glutamate transporters in the CNS: molecular mechanism, pre-steady-state kinetics, and their impact on synaptic signaling. *J Membr Biol* 203: 1-20.

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. *Trans Am Ophthalmol Soc* 101: 163-169; discussion 169-171.

Grunert U, Martin PR & Wassle H. (1994) Immunocytochemical analysis of bipolar cells in the macaque monkey retina. *J Comp Neurol* 348: 607-627.

Grunwald JE, Piltz J, Patel N, Bose S & Riva CE. (1993) Effect of aging on retinal macular microcirculation: a blue field simulation study. *Invest Ophthalmol Vis Sci* 34: 3609-3613.

Gunhan-Agar E, Kahn D & Chalupa LM. (2000) Segregation of on and off bipolar cell axonal arbors in the absence of retinal ganglion cells. *J Neurosci* 20: 306-314.

Guo D, Bi H, Wu Q, Wang D & Cui Y. (2013) Zinc oxide nanoparticles induce rat retinal ganglion cell damage through bcl-2, caspase-9 and caspase-12 pathways. *J Nanosci Nanotechnol* 13: 3769-3777.

Haberecht MF, Mitchell CK, Lo GJ & Redburn DA. (1997) N-methyl-D-aspartate-mediated glutamate toxicity in the developing rabbit retina. *J Neurosci Res* 47: 416-426.

Hafez AS, Bizzarro RL & Lesk MR. (2003) Evaluation of optic nerve head and peripapillary retinal blood flow in glaucoma patients, ocular hypertensives, and normal subjects. *Am J Ophthalmol* 136: 1022-1031.

Ham RG. (1965) Clonal Growth of Mammalian Cells in a Chemically Defined, Synthetic Medium. *Proc Natl Acad Sci U S A* 53: 288-293.

Han Y, Xu J, Li Z & Yang Z. (2013) Neuroprotective effect of leukemia inhibitory factor on antimycin A-induced oxidative injury in differentiated PC12 cells. *J Mol Neurosci* 50: 577-585.

Hancock HA, Guidry C, Read RW, Ready EL & Kraft TW. (2005) Acute aminoglycoside retinal toxicity in vivo and in vitro. *Invest Ophthalmol Vis Sci* 46: 4804-4808.

Hangai M, Yoshimura N, Yoshida M, Yabuuchi K & Honda Y. (1995) Interleukin-1 gene expression in transient retinal ischemia in the rat. *Invest Ophthalmol Vis Sci* 36: 571-578.

Hanninen VA, Pantcheva MB, Freeman EE, Poulin NR & Grosskreutz CL. (2002) Activation of caspase 9 in a rat model of experimental glaucoma. *Curr Eye Res* 25: 389-395.

Hara N, Alkanani AK, Dinarello CA & Zipris D. (2014) Histone deacetylase inhibitor suppresses virus-induced proinflammatory responses and type 1 diabetes. *J Mol Med (Berl)* 92: 93-102.

Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K*, et al.* (2007) The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. *J Clin Invest* 117: 1763-1770.

Harper MM, Adamson L, Blits B, Bunge MB, Grozdanic SD & Sakaguchi DS. (2009) Brain-derived neurotrophic factor released from engineered mesenchymal stem cells attenuates glutamate- and hydrogen peroxide-mediated death of staurosporine-differentiated RGC-5 cells. *Exp Eye Res* 89: 538-548.

Harris A, Ciulla TA, Chung HS & Martin B. (1998) Regulation of retinal and optic nerve blood flow. *Arch Ophthalmol* 116: 1491-1495.

Hartl FU. (1996) Molecular chaperones in cellular protein folding. *Nature* 381: 571-579.

Haverkamp S & Wassle H. (2000) Immunocytochemical analysis of the mouse retina. *J Comp Neurol* 424: 1-23.

He F, Kumar A & Song ZH. (2012) Heat shock protein 90 is an essential molecular chaperone for CB2 cannabinoid receptor-mediated signaling in trabecular meshwork cells. *Mol Vis* 18: 2839-2846.

He L, Yang H, Gardiner SK, Williams G, Hardin C, Strouthidis NG*, et al.* (2014) Longitudinal detection of optic nerve head changes by spectral domain optical coherence tomography in early experimental glaucoma. *Invest Ophthalmol Vis Sci* 55: 574-586.

He XY, Chen ZZ, Cai YQ, Xu G, Shang JH, Kou SB*, et al.* (2011) Expression of cytokines in rat brain with focal cerebral ischemia after grafting with bone marrow stromal cells and endothelial progenitor cells. *Cytotherapy* 13: 46-53.

Hebbes TR, Thorne AW & Crane-Robinson C. (1988) A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J* 7: 1395-1402.

Heier JS, Antoszyk AN, Pavan PR, Leff SR, Rosenfeld PJ, Ciulla TA*, et al.* (2006) Ranibizumab for treatment of neovascular age-related macular degeneration: a phase I/II multicenter, controlled, multidose study. *Ophthalmology* 113: 633 e631-634.

Heijl A, Bengtsson B & Oskarsdottir SE. (2013) Prevalence and severity of undetected manifest glaucoma: results from the early manifest glaucoma trial screening. *Ophthalmology* 120: 1541-1545.

Heijl A, Leske MC, Bengtsson B, Hyman L, Bengtsson B, Hussein M & Early Manifest Glaucoma Trial G. (2002) Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial. *Arch Ophthalmol* 120: 1268-1279.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G & Schaper F. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 374: 1-20.

Hengartner MO. (2000) The biochemistry of apoptosis. *Nature* 407: 770-776.

Hernandez MR. (2000) The optic nerve head in glaucoma: Role of astrocytes in tissue remodeling. *Prog Retin Eye Res* 19: 297-321.

Herrero-Mendez A, Almeida A, Fernandez E, Maestre C, Moncada S & Bolanos JP. (2009) The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol* 11: 747-752.

Hettich C, Wilker S, Mentlein R, Lucius R, Roider J & Klettner A. (2014) The retinal pigment epithelium (RPE) induces FasL and reduces iNOS and Cox2 in primary monocytes. *Graefes Arch Clin Exp Ophthalmol*: 1-8.

Hohenauer T, Berking C, Schmidt A, Haferkamp S, Senft D, Kammerbauer C*, et al.* (2013) The neural crest transcription factor Brn3a is expressed in melanoma and required for cell cycle progression and survival. *EMBO Mol Med* 5: 919-934.

Hollander DA, Sarfarazi M, Stoilov I, Wood IS, Fredrick DR & Alvarado JA. (2006) Genotype and phenotype correlations in congenital glaucoma. *Trans Am Ophthalmol Soc* 104: 183-195.

Hollows FC & Graham PA. (1966) Intra-Ocular Pressure Glaucoma and Glaucoma Suspects in a Defined Population. *Br J Ophthalmol* 50: 570-580.

Hopkins JM & Bunge RP. (1991) Regeneration of axons from adult human retina in vitro. *Exp Neurol* 112: 243-251.

Horton AR, Barlett PF, Pennica D & Davies AM. (1998) Cytokines promote the survival of mouse cranial sensory neurones at different developmental stages. *Eur J Neurosci* 10: 673-679.

Hsieh J, Nakashima K, Kuwabara T, Mejia E & Gage FH. (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A* 101: 16659-16664.

Hsieh JY, Wang HW, Chang SJ, Liao KH, Lee IH, Lin WS*, et al.* (2013) Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. *PLoS One* 8: e72604-77215.

Huang W, Fileta J, Guo Y & Grosskreutz CL. (2006) Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. *Curr Eye Res* 31: 265-271.

Hudson CD, Podesta J, Henderson D, Latchman DS & Budhram-Mahadeo V. (2004) Coexpression of Brn-3a POU protein with p53 in a population of neuronal progenitor cells is associated with differentiation and protection against apoptosis. *J Neurosci Res* 78: 803-814.

Iadecola C & Nedergaard M. (2007) Glial regulation of the cerebral microvasculature. *Nat Neurosci* 10: 1369-1376.

Ikeda K, Iwasaki Y, Tagaya N, Shiojima T & Kinoshita M. (1995) Neuroprotective effect of cholinergic differentiation factor/leukemia inhibitory factor on wobbler murine motor neuron disease. *Muscle Nerve* 18: 1344-1347.

Ishii Y, Oya T, Zheng L, Gao Z, Kawaguchi M, Sabit H*, et al.* (2006) Mouse brains deficient in neuronal PDGF receptor-beta develop normally but are vulnerable to injury. *J Neurochem* 98: 588-600.

Issa JP. (2000) CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol* 249: 101-118.

Jackson GG & Arcieri G. (1971) Ototoxicity of gentamicin in man: a survey and controlled analysis of clinical experience in the United States. *J Infect Dis* 124 Suppl: S130-137.

Jaenisch R & Bird A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 33 Suppl: 245-254.

Jamaladdin S, Kelly RD, O'Regan L, Dovey OM, Hodson GE, Millard CJ*, et al.* (2014) Histone deacetylase (HDAC) 1 and 2 are essential for accurate cell division and the pluripotency of embryonic stem cells. *Proc Natl Acad Sci U S A* 111: 9840-9845.

Jarrett SG, Lin H, Godley BF & Boulton ME. (2008) Mitochondrial DNA damage and its potential role in retinal degeneration. *Prog Retin Eye Res* 27: 596-607.

Jayme D, Watanabe T & Shimada T. (1997) Basal medium development for serum-free culture: a historical perspective. *Cytotechnology* 23: 95-101.

Jenuwein T & Allis CD. (2001) Translating the histone code. *Science* 293: 1074-1080.

Jin KL, Mao XO & Greenberg DA. (2000a) Vascular endothelial growth factor rescues HN33 neural cells from death induced by serum withdrawal. *J Mol Neurosci* 14: 197-203.

Jin KL, Mao XO & Greenberg DA. (2000b) Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci U S A* 97: 10242-10247.

Jo N, Mailhos C, Ju M, Cheung E, Bradley J, Nishijima K*, et al.* (2006) Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. *Am J Pathol* 168: 2036-2053.

John SW, Smith RS, Savinova OV, Hawes NL, Chang B, Turnbull D*, et al.* (1998) Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice. *Invest Ophthalmol Vis Sci* 39: 951-962.

Johnson EC, Deppmeier LM, Wentzien SK, Hsu I & Morrison JC. (2000) Chronology of optic nerve head and retinal responses to elevated intraocular pressure. *Invest Ophthalmol Vis Sci* 41: 431-442.

Johnson EC, Doser TA, Cepurna WO, Dyck JA, Jia L, Guo Y*, et al.* (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: 504-518.

Johnson M. (2006) 'What controls aqueous humour outflow resistance?'. *Exp Eye Res* 82: 545-557.

Johnson PT, Williams RR, Cusato K & Reese BE. (1999) Rods and cones project to the inner plexiform layer during development. *J Comp Neurol* 414: 1-12.

Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI & Martin KR. (2010) Neuroprotective effects of intravitreal mesenchymal stem cell transplantation in experimental glaucoma. *Invest Ophthalmol Vis Sci* 51: 2051-2059.

Johnson TV, DeKorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B*, et al.* (2014) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. *Brain* 137: 503-519.

Johnson TV & Martin KR. (2008) Development and characterization of an adult retinal explant organotypic tissue culture system as an in vitro intraocular stem cell transplantation model. *Invest Ophthalmol Vis Sci* 49: 3503-3512.

Joly S, Lange C, Thiersch M, Samardzija M & Grimm C. (2008) Leukemia inhibitory factor extends the lifespan of injured photoreceptors in vivo. *J Neurosci* 28: 13765-13774.

Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS & Wu CW. (2000) Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem* 275: 20436-20443.

Kallberg ME, Brooks DE, Gelatt KN, Garcia-Sanchez GA, Szabo NJ & Lambrou GN. (2007) Endothelin-1, nitric oxide, and glutamate in the normal and glaucomatous dog eye. *Vet Ophthalmol* 10 Suppl 1: 46-52.

Kanamori A, Naka M, Fukuda M, Nakamura M & Negi A. (2009) Latanoprost protects rat retinal ganglion cells from apoptosis in vitro and in vivo. *Exp Eye Res* 88: 535-541.

Kanamoto T, Rimayanti U, H O & Kiuchi Y. (2011) Platelet-derived growth factor receptor alpha is associated with oxidative stress-induced retinal cell death. *Curr Eye Res* 36: 336-340.

Kanter ED & Brucker AJ. (1995) Aminoglycoside macular infarction in association with gentamicin-soaked collagen corneal shield. *Arch Ophthalmol* 113: 1359-1360.

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV*, et al.* (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 5: 74-80.

Kass MA, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP*, et al.* (2002) The Ocular Hypertension Treatment Study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch Ophthalmol* 120: 701-713.

Kazlauskas A. (2000) A new member of an old family. *Nat Cell Biol* 2: E78-79.

Kern TJ. (1997) Rabbit and rodent ophthalmology. *Seminars in Avian and Exotic Pet Medicine* 6: 138-145.

Kerrigan LA, Zack DJ, Quigley HA, Smith SD & Pease ME. (1997) TUNEL-positive ganglion cells in human primary open-angle glaucoma. *Arch Ophthalmol* 115: 1031-1035.

Khachik F, Bernstein PS & Garland DL. (1997) Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Invest Ophthalmol Vis Sci* 38: 1802-1811.

Khaibullina AA, Rosenstein JM & Krum JM. (2004) Vascular endothelial growth factor promotes neurite maturation in primary CNS neuronal cultures. *Brain Res Dev Brain Res* 148: 59-68.

Kim I, Ryan AM, Rohan R, Amano S, Agular S, Miller JW & Adamis AP. (1999) Constitutive expression of VEGF, VEGFR-1, and VEGFR-2 in normal eyes. *Invest Ophthalmol Vis Sci* 40: 2115-2121.

Kim JH, Nam KH, Kwon OS, Kim IG, Bustin M, Choy HE & Park SC. (2002) Histone cross-linking by transglutaminase. *Biochem Biophys Res Commun* 293: 1453-1457.

Kim KK, Adelstein RS & Kawamoto S. (2009) Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. *J Biol Chem* 284: 31052-31061.

Kim SU & Takahashi H. (1988) Tissue culture study of adult human retina neurons. *Invest Ophthalmol Vis Sci* 29: 1372-1379.

Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S & Epstein SE. (2004) Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 94: 678-685.

Kinnear A, Lauber JK & Boyd TA. (1974) Genesis of light-induced avian glaucoma. *Invest Ophthalmol* 13: 872-875.

Klocker N, Braunling F, Isenmann S & Bahr M. (1997) In vivo neurotrophic effects of GDNF on axotomized retinal ganglion cells. *Neuroreport* 8: 3439-3442.

Klocker N, Kermer P, Weishaupt JH, Labes M, Ankerhold R & Bahr M. (2000) Brain-derived neurotrophic factor-mediated neuroprotection of adult rat retinal ganglion cells in vivo does not exclusively depend on phosphatidyl-inositol-3'-kinase/protein kinase B signaling. *J Neurosci* 20: 6962-6967.

Knoblach SM, Fan L & Faden AI. (1999) Early neuronal expression of tumor necrosis factor-alpha after experimental brain injury contributes to neurological impairment. *J Neuroimmunol* 95: 115-125.

Knott RM, Robertson M, Muckersie E, Folefac VA, Fairhurst FE, Wileman SM & Forrester JV. (1999) A model system for the study of human retinal

angiogenesis: activation of monocytes and endothelial cells and the association with the expression of the monocarboxylate transporter type 1 (MCT-1). *Diabetologia* 42: 870-877.

Knowlton AA, Kapadia S, Torre-Amione G, Durand JB, Bies R, Young J & Mann DL. (1998) Differential expression of heat shock proteins in normal and failing human hearts. *J Mol Cell Cardiol* 30: 811-818.

Kobayakawa S, Hiratsuka Y, Watabe Y, Murakami A & Tochikubo T. (2010) Comparison of the influence of intracameral gentamicin, gatifloxacin, and moxifloxacin on the corneal endothelium in a rabbit model. *Jpn J Ophthalmol* 54: 481-485.

Koh JY & Choi DW. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* 20: 83-90.

Koizumi A, Zeck G, Ben Y, Masland RH & Jakobs TC. (2007) Organotypic culture of physiologically functional adult mammalian retinas. *PLoS One* 2: e221-229.

Konsoula Z & Barile FA. (2012) Epigenetic histone acetylation and deacetylation mechanisms in experimental models of neurodegenerative disorders. *J Pharmacol Toxicol Methods* 66: 215-220.

Kook SY, Jeong H, Kang MJ, Park R, Shin HJ, Han SH*, et al.* (2014) Crucial role of calbindin-D28k in the pathogenesis of Alzheimer's disease mouse model. *Cell Death Differ* 21: 1575-1587.

Korf HW, White BH, Schaad NC & Klein DC. (1992) Recoverin in pineal organs and retinae of various vertebrate species including man. *Brain Res* 595: 57-66.

Kovacs JJ, Murphy PJ, Gaillard S, Zhao X, Wu JT, Nicchitta CV*, et al.* (2005) HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Mol Cell* 18: 601-607.

Krammer PH, Arnold R & Lavrik IN. (2007) Life and death in peripheral T cells. *Nature Rev Immunol* 7: 532-542.

Krishnamoorthy RR, Agarwal P, Prasanna G, Vopat K, Lambert W, Sheedlo HJ*, et al.* (2001) Characterization of a transformed rat retinal ganglion cell line. *Brain Res Mol Brain Res* 86: 1-12.

Krishnamoorthy RR, Clark AF, Daudt D, Vishwanatha JK & Yorio T. (2013) A forensic path to RGC-5 cell line identification: lessons learned. *Invest Ophthalmol Vis Sci* 54: 5712-5719.

Krizova D, Vokrojova M, Liehneova K & Studeny P. (2014) Treatment of Corneal Neovascularization Using Anti-VEGF Bevacizumab. *J Ophthalmol* 2014: 178132.

Kuffler SW. (1953) Discharge patterns and functional organization of mammalian retina. *J Neurophysiol* 16: 37-68.

Kugler P & Beyer A. (2003) Expression of glutamate transporters in human and rat retina and rat optic nerve. *Histochem Cell Biol* 120: 199-212.

Kupcova-Skalnikova H. (2013) Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie* 95: 2196-2211.

Lagreze WA, Knorle R, Bach M & Feuerstein TJ. (1998) Memantine is neuroprotective in a rat model of pressure-induced retinal ischemia. *Invest Ophthalmol Vis Sci* 39: 1063-1066.

Lambrecht HG & Koch KW. (1992) Recoverin, a novel calcium-binding protein from vertebrate photoreceptors. *Biochim Biophys Acta* 1160: 63-66.

Larsson OM, Hertz L & Schousboe A. (1980) GABA uptake in astrocytes in primary cultures: coupling with two sodium ions. *J Neurosci Res* 5: 469-477.

Leary AG, Wong GG, Clark SC, Smith AG & Ogawa M. (1990) Leukemia inhibitory factor differentiation-inhibiting activity/human interleukin for DA cells augments proliferation of human hematopoietic stem cells. *Blood* 75: 1960-1964.

Lee D, Kim KY, Noh YH, Chai S, Lindsey JD, Ellisman MH*, et al.* (2012) Brimonidine blocks glutamate excitotoxicity-induced oxidative stress and preserves mitochondrial transcription factor a in ischemic retinal injury. *PLoS One* 7: e47098-47110.

Lee DY, Hayes JJ, Pruss D & Wolffe AP. (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell* 72: 73-84.

Lee P. (1989) Ophthalmic findings in laboratory animals. *Anim Eye Res*: 1–12.

Lee W, Tillo D, Bray N, Morse RH, Davis RW, Hughes TR & Nislow C. (2007) A high-resolution atlas of nucleosome occupancy in yeast. *Nat Genet* 39: 1235-1244.

Lei H & Kazlauskas A. (2008) Focus on molecules: platelet-derived growth factor C, PDGF-C. *Exp Eye Res* 86: 711-712.

Lei H, Rheaume MA & Kazlauskas A. (2010) Recent developments in our understanding of how platelet-derived growth factor (PDGF) and its receptors contribute to proliferative vitreoretinopathy. *Exp Eye Res* 90: 376-381.

Leibinger M, Muller A, Andreadaki A, Hauk TG, Kirsch M & Fischer D. (2009) Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. *J Neurosci* 29: 14334-14341.

Leng Y, Marinova Z, Reis-Fernandes MA, Nau H & Chuang DM. (2010) Potent neuroprotective effects of novel structural derivatives of valproic acid: potential roles of HDAC inhibition and HSP70 induction. *Neurosci Lett* 476: 127-132.

Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P*, et al.* (2002) The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc Natl Acad Sci U S A* 99: 2995-3000.

Leske MC. (2009) Ocular perfusion pressure and glaucoma: clinical trial and epidemiologic findings. *Curr Opin Ophthalmol* 20: 73-78.

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

Leske MC, Wu SY, Nemesure B & Hennis A. (2002) Incident open-angle glaucoma and blood pressure. *Arch Ophthalmol* 120: 954-959.

Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E & Betsholtz C. (1994) Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8: 1875-1887.

Levi G & Patrizio M. (1992) Astrocyte heterogeneity: endogenous amino acid levels and release evoked by non-N-methyl-D-aspartate receptor agonists and by potassium-induced swelling in type-1 and type-2 astrocytes. *J Neurochem* 58: 1943-1952.

Levin LA & Louhab A. (1996) Apoptosis of retinal ganglion cells in anterior ischemic optic neuropathy. *Arch Ophthalmol* 114: 488-491.

Levkovitch-Verbin H, Martin KR, Quigley HA, Baumrind LA, Pease ME & Valenta D. (2002a) Measurement of amino acid levels in the vitreous humor of rats after chronic intraocular pressure elevation or optic nerve transection. *J Glaucoma* 11: 396-405.

Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA & Pease ME. (2002b) Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci* 43: 402-410.

Levkovitch-Verbin H, Quigley HA, Martin KRG, Zack DJ, Pease ME & Valenta DF. (2003) A model to study differences between primary and secondary degeneration of retinal ganglion cells in rats by partial optic nerve transection. *Invest Ophth Vis Sci* 44: 3388-3393.

Lewis PR, Phillips TG & Sassani JW. (1999) Topical therapies for glaucoma: what family physicians need to know. *Am Fam Physician* 59: 1871-1879, 1882.

Li N, Li XR & Yuan JQ. (2009) Effects of bone-marrow mesenchymal stem cells transplanted into vitreous cavity of rat injured by ischemia/reperfusion. *Graefes Arch Clin Exp Ophthalmol* 247: 503-514.

Li N, Wang P, Ma XL, Wang J, Zhao LJ, Du L*, et al.* (2014) Effect of bone marrow stromal cell transplantation on neurologic function and expression of VEGF in rats with focal cerebral ischemia. *Mol Med Rep* 10: 2299-2305.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES & Wang X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479-489.

Li X & Eriksson U. (2003) Novel PDGF family members: PDGF-C and PDGF-D. *Cytokine Growth Factor Rev* 14: 91-98.

Li Y, Schlamp CL & Nickells RW. (1999) Experimental induction of retinal ganglion cell death in adult mice. *Invest Ophthalmol Vis Sci* 40: 1004-1008.

Libby RT, Li Y, Savinova OV, Barter J, Smith RS, Nickells RW & John SW. (2005) Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. *PLoS Genet* 1: 17-26.

Liegl R, Koenig S, Siedlecki J, Haritoglou C, Kampik A & Kernt M. (2014) Temsirolimus inhibits proliferation and migration in retinal pigment epithelial and endothelial cells via mTOR inhibition and decreases VEGF and PDGF expression. *PLoS One* 9: e88203-88213.

Lillycrop KA, Hoile SP, Grenfell L & Burdge GC. (2014) DNA methylation, ageing and the influence of early life nutrition. *Proc Nutr Soc* 73: 413-421.

Lin HS, Hu CY, Chan HY, Liew YY, Huang HP, Lepescheux L*, et al.* (2007) Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. *Br J Pharmacol* 150: 862-872.

Lind D, Franken S, Kappler J, Jankowski J & Schilling K. (2005) Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. *J Neurosci Res* 79: 295-302.

Lindquist S & Craig EA. (1988) The heat-shock proteins. *Annu Rev Genet* 22: 631-677.

Lipton SA & Nicotera P. (1998) Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell calcium* 23: 165-171.

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. (2001) Nitric oxide synthase-2 in human optic nerve head astrocytes induced by elevated pressure in vitro. *Arch Ophthalmol* 119: 240-245.

Lo AC, Woo TT, Wong RL & Wong D. (2011) Apoptosis and other cell death mechanisms after retinal detachment: implications for photoreceptor rescue. *Ophthalmologica* 226 Suppl 1: 10-17.

Lorch Y, Zhang M & Kornberg RD. (1999) Histone octamer transfer by a chromatin-remodeling complex. *Cell* 96: 389-392.

Lowe RF. (1970) Aetiology of Anatomical Basis for Primary Angle-Closure Glaucoma - Biometrical Comparisons between Normal Eyes and Eyes with Primary Angle-Closure Glaucoma. *Br J Ophthalmol* 54: 161-170.

Lu L, Wang W, Peng Y, Li J, Wang L & Wang X. (2014) Electrophysiology and pharmacology of tandem domain potassium channel TREK-1 related BDNF synthesis in rat astrocytes. *Naunyn Schmiedebergs Arch Pharmacol* 387: 303-312.

Lucas DR & Newhouse JP. (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. *AMA Arch Ophthalmol* 58: 193-201.

Lucas DR & Trowell OA. (1958) In vitro culture of the eye and the retina of the mouse and rat. *J Embryol Exp Morphol* 6: 178-182.

Luger K, Mader AW, Richmond RK, Sargent DF & Richmond TJ. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389: 251-260.

Lukas TJ & Wang AL. (2012) Isolation and culture of astrocytes from the retina and optic nerve. *Methods Mol Biol* 814: 105-115.

Luo J, Su F, Chen D, Shiloh A & Gu W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* 408: 377-381.

Lye MH, Jakobs TC, Masland RH & Koizumi A. (2007) Organotypic culture of adult rabbit retina. *J Vis Exp*: 190-195.

Ma N. 2013. Development of techniques for analysis of the human retinal ganglion cell transcriptome: application to the role of calcium in RGC death in glaucoma. In *School of Pharmacy*, 158. The University of East Anglia.

Ma Y, Liu W, Wang Y, Chao X, Qu Y, Wang K & Fei Z. (2011) VEGF protects rat cortical neurons from mechanical trauma injury induced apoptosis via the MEK/ERK pathway. *Brain Res Bull* 86: 441-446.

Magistretti PJ, Pellerin L, Rothman DL & Shulman RG. (1999) Energy on demand. *Science* 283: 496-497.

Maher P & Hanneken A. (2005) Flavonoids protect retinal ganglion cells from oxidative stress-induced death. *Invest Ophthalmol Vis Sci* 46: 4796-4803.

Majumder A, Banerjee S, Harrill JA, Machacek DW, Mohamad O, Bacanamwo M*, et al.* (2012) Neurotrophic effects of leukemia inhibitory factor on neural cells derived from human embryonic stem cells. *Stem Cells* 30: 2387-2399.

Manabe S, Kashii S, Honda Y, Yamamoto R, Katsuki H & Akaike A. (2002) Quantification of axotomized ganglion cell death by explant culture of the rat retina. *Neurosci Lett* 334: 33-36.

Marcaggi P & Attwell D. (2004) Role of glial amino acid transporters in synaptic transmission and brain energetics. *Glia* 47: 217-225.

Marcuccilli CJ, Mathur SK, Morimoto RI & Miller RJ. (1996) Regulatory differences in the stress response of hippocampal neurons and glial cells after heat shock. *J Neurosci* 16: 478-485.

Marcus MW, de Vries MM, Junoy Montolio FG & Jansonius NM. (2011) Myopia as a risk factor for open-angle glaucoma: a systematic review and meta-analysis. *Ophthalmology* 118: 1989-1994.

Marinova Z, Ren M, Wendland JR, Leng Y, Liang MH, Yasuda S*, et al.* (2009) Valproic acid induces functional heat-shock protein 70 via Class I histone deacetylase inhibition in cortical neurons: a potential role of Sp1 acetylation. *J Neurochem* 111: 976-987.

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. *Invest Ophthalmol Vis Sci* 43: 2236-2243.

Martinez A & Sanchez M. (2005) Predictive value of colour Doppler imaging in a prospective study of visual field progression in primary open-angle glaucoma. *Acta Ophthalmol Scand* 83: 716-722.

Martinou JC, Martinou I & Kato AC. (1992) Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. *Neuron* 8: 737-744.

Masland RH. (2001) The fundamental plan of the retina. *Nat Neurosci* 4: 877-886.

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

Mavrich TN, Jiang C, Ioshikhes IP, Li X, Venters BJ, Zanton SJ*, et al.* (2008) Nucleosome organization in the Drosophila genome. *Nature* 453: 358-362.

May CA, Hayreh SS, Furuyoshi N, Ossoinig K, Kaufman PL & Lutjen-Drecoll E. (1997) Choroidal ganglion cell plexus and retinal vasculature in monkeys with laser-induced glaucoma. *Ophthalmologica* 211: 161-171.

Mayer EJ, Carter DA, Ren Y, Hughes EH, Rice CM, Halfpenny CA*, et al.* (2005) Neural progenitor cells from postmortem adult human retina. *Br J Ophthalmol* 89: 102-106.

McKee AE, Minet E, Stern C, Riahi S, Stiles CD & Silver PA. (2005) A genome-wide in situ hybridization map of RNA-binding proteins reveals anatomically restricted expression in the developing mouse brain. *BMC Dev Biol* 5: 14-23.

McKernan DP, Caplis C, Donovan M, O'Brien C J & Cotter TG. (2006) Age-dependent susceptibility of the retinal ganglion cell layer to cell death. *Invest Ophthalmol Vis Sci* 47: 807-814.

McKinnon SJ, Lehman DM, Kerrigan-Baumrind LA, Merges CA, Pease ME, Kerrigan DF*, et al.* (2002) Caspase activation and amyloid precursor protein cleavage in rat ocular hypertension. *Invest Ophthalmol Vis Sci* 43: 1077-1087.

Mekada A, Sasahara M, Yamada E, Kani K & Hazama F. (1998) Platelet-derived growth factor B-chain expression in the rat retina and optic nerve: distribution and changes after transection of the optic nerve. *Vision Res* 38: 3031-3039.

Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G*, et al.* (2003) In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res* 9: 327-337.

Mey J & Thanos S. (1993) Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. *Brain Res* 602: 304-317.

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.

Mi H, Haeberle H & Barres BA. (2001) Induction of astrocyte differentiation by endothelial cells. *J Neurosci* 21: 1538-1547.

Milam AH, Dacey DM & Dizhoor AM. (1993) Recoverin immunoreactivity in mammalian cone bipolar cells. *Vis Neurosci* 10: 1-12.

Miller ED, Tran MN, Wong GK, Oakley DM & Wong RO. (1999) Morphological differentiation of bipolar cells in the ferret retina. *Vis Neurosci* 16: 1133-1144.

Minami M, Kuraishi Y, Yabuuchi K, Yamazaki A & Satoh M. (1992) Induction of interleukin-1 beta mRNA in rat brain after transient forebrain ischemia. *J Neurochem* 58: 390-392.

Minckler DS, Bunt AH & Johanson GW. (1977) Orthograde and retrograde axoplasmic transport during acute ocular hypertension in the monkey. *Invest Ophthalmol Vis Sci* 16: 426-441.

Montero M, Gonzalez B & Zimmer J. (2009) Immunotoxic depletion of microglia in mouse hippocampal slice cultures enhances ischemia-like neurodegeneration. *Brain Res* 1291: 140-152.

Mudhar HS, Pollock RA, Wang C, Stiles CD & Richardson WD. (1993) PDGF and its receptors in the developing rodent retina and optic nerve. *Development* 118: 539-552.

Mullen RJ, Buck CR & Smith AM. (1992) NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116: 201-211.

Murphy PJ, Morishima Y, Kovacs JJ, Yao TP & Pratt WB. (2005) Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone. *J Biol Chem* 280: 33792-33799.

Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, Salinas-Navarro M*, et al.* (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: 3860-3868.

Nagashima M, Fujikawa C, Mawatari K, Mori Y & Kato S. (2011) HSP70, the earliest-induced gene in the zebrafish retina during optic nerve regeneration: its role in cell survival. *Neurochem Int* 58: 888-895.

Nakanishi M, Niidome T, Matsuda S, Akaike A, Kihara T & Sugimoto H. (2007) Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells. *Eur J Neurosci* 25: 649-658.

Nakanishi Y, Nakamura M, Mukuno H, Kanamori A, Seigel GM & Negi A. (2006) Latanoprost rescues retinal neuro-glial cells from apoptosis by inhibiting caspase-3, which is mediated by p44/p42 mitogen-activated protein kinase. *Exp Eye Res* 83: 1108-1117.

Nakhai H, Sel S, Favor J, Mendoza-Torres L, Paulsen F, Duncker GI & Schmid RM. (2007) Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. *Development* 134: 1151-1160.

Nasef A, Mazurier C, Bouchet S, Francois S, Chapel A, Thierry D*, et al.* (2008) Leukemia inhibitory factor: Role in human mesenchymal stem cells mediated immunosuppression. *Cell Immunol* 253: 16-22.

Neufeld AH, Hernandez MR & Gonzalez M. (1997) Nitric oxide synthase in the human glaucomatous optic nerve head. *Arch Ophthalmol* 115: 497-503.

Nickells RW. (1999) Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death. *Surv Ophthalmol* 43 Suppl 1: S151-161.

Nishijima K, Ng YS, Zhong L, Bradley J, Schubert W, Jo N*, et al.* (2007) Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *Am J Pathol* 171: 53-67.

Nishimura Y, Murakami S, Ashikawa Y, Sasagawa S, Umemoto N, Shimada Y & Tanaka T. (2014) Zebrafish as a systems toxicology model for developmental neurotoxicity testing. *Congenit Anom (Kyoto)* Epub ahead of print.

Niyadurupola N, Sidaway P, Ma N, Rhodes JD, Broadway DC & Sanderson J. (2013) P2X7 receptor activation mediates retinal ganglion cell death in a human retina model of ischemic neurodegeneration. *Invest Ophthalmol Vis Sci* 54: 2163-2170.

Niyadurupola N, Sidaway P, Osborne A, Broadway DC & Sanderson J. (2011) The development of human organotypic retinal cultures (HORCs) to study retinal neurodegeneration. *Br J Ophthalmol* 95: 720-726.

Noone P, Parsons TM, Pattison JR, Slack RC, Garfield-Davies D & Hughes K. (1974) Experience in monitoring gentamicin therapy during treatment of serious gram-negative sepsis. *Br Med J* 1: 477-481.

Nural-Guvener HF, Zakharova L, Nimlos J, Popovic S, Mastroeni D & Gaballa MA. (2014) HDAC class I inhibitor, Mocetinostat, reverses cardiac fibrosis in heart failure and diminishes CD90+ cardiac myofibroblast activation. *Fibrogenesis Tissue Repair* 7: 10-26.

Oguni M, Tanaka O, Tamura H, Shinohara H, Kato K & Setogawa T. (1995) Ontogeny of copper-zinc and manganese superoxide dismutase in the developing rat retina: immunohistochemical and immunochemical study. *Ophthalmic Res* 27: 227-233.

Okisaka S, Murakami A, Mizukawa A & Ito J. (1997) Apoptosis in retinal ganglion cell decrease in human glaucomatous eyes. *Jpn J Ophthalmol* 41: 84-88.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K*, et al.* (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 28: 131-138.

Osborne NN, DeSantis L, Bae JH, Ugarte M, Wood JP, Nash MS & Chidlow G. (1999) Topically applied betaxolol attenuates NMDA-induced toxicity to ganglion cells and the effects of ischaemia to the retina. *Exp Eye Res* 69: 331-342.

Osborne NN & Larsen AK. (1996) Antigens associated with specific retinal cells are affected by ischaemia caused by raised intraocular pressure: effect of glutamate antagonists. *Neurochem Int* 29: 263-270.

Otori Y, Kusaka S, Kawasaki A, Morimura H, Miki A & Tano Y. (2003) Protective effect of nilvadipine against glutamate neurotoxicity in purified retinal ganglion cells. *Brain Res* 961: 213-219.

Otori Y, Shimada S, Tanaka K, Ishimoto I, Tano Y & Tohyama M. (1994) Marked increase in glutamate-aspartate transporter (GLAST/GluT-1) mRNA following transient retinal ischemia. *Brain Res Mol Brain Res* 27: 310-314.

Ozawa Y, Yuki K, Yamagishi R, Tsubota K & Aihara M. (2013) Renin-angiotensin system involvement in the oxidative stress-induced neurodegeneration of cultured retinal ganglion cells. *Jpn J Ophthalmol* 57: 126-132.

Pacal M & Bremner R. (2014) Induction of the ganglion cell differentiation program in human retinal progenitors before cell cycle exit. *Dev Dyn* 243: 712-729.

Pang IH, Johnson EC, Jia L, Cepurna WO, Shepard AR, Hellberg MR*, et al.* (2005) Evaluation of inducible nitric oxide synthase in glaucomatous optic neuropathy and pressure-induced optic nerve damage. *Invest Ophthalmol Vis Sci* 46: 1313-1321.

Pang IH, Zeng H, Fleenor DL & Clark AF. (2007) Pigment epithelium-derived factor protects retinal ganglion cells. *BMC Neurosci* 8: 11-23.

Pang JJ, Paul DL & Wu SM. (2013) Survey on amacrine cells coupling to retrograde-identified ganglion cells in the mouse retina. *Invest Ophthalmol Vis Sci* 54: 5151-5162.

Pang M, Kothapally J, Mao H, Tolbert E, Ponnusamy M, Chin YE & Zhuang S. (2009) Inhibition of histone deacetylase activity attenuates renal fibroblast activation and interstitial fibrosis in obstructive nephropathy. *Am J Physiol Renal Physiol* 297: F996-1005.

Paquet-Durand F, Silva J, Talukdar T, Johnson LE, Azadi S, van Veen T*, et al.* (2007) Excessive activation of poly(ADP-ribose) polymerase contributes to inherited photoreceptor degeneration in the retinal degeneration 1 mouse. *J Neurosci* 27: 10311-10319.

Parekkadan B, van Poll D, Megeed Z, Kobayashi N, Tilles AW, Berthiaume F & Yarmush ML. (2007) Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. *Biochem Biophys Res Commun* 363: 247-252.

Park HY, Kim JH & Park CK. (2014) Neuronal cell death in the inner retina and the influence of vascular endothelial growth factor inhibition in a diabetic rat model. *Am J Pathol* 184: 1752-1762.

Park JI, Strock CJ, Ball DW & Nelkin BD. (2003) The Ras/Raf/MEK/extracellular signal-regulated kinase pathway induces autocrine-paracrine growth inhibition via the leukemia inhibitory factor/JAK/STAT pathway. *Mol Cell Biol* 23: 543-554.

Pellerin L & Magistretti PJ. (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A* 91: 10625-10629.

Pelzel HR, Schlamp CL & Nickells RW. (2010) Histone H4 deacetylation plays a critical role in early gene silencing during neuronal apoptosis. *BMC Neurosci* 11-32: 62.

Pelzel HR, Schlamp CL, Waclawski M, Shaw MK & Nickells RW. (2012) Silencing of Fem1cR3 gene expression in the DBA/2J mouse precedes retinal ganglion cell death and is associated with histone deacetylase activity. *Invest Ophthalmol Vis Sci* 53: 1428-1435.

Penha FM, Rodrigues EB, Maia M, Furlani BA, Regatieri C, Melo GB*, et al.* (2010) Retinal and ocular toxicity in ocular application of drugs and chemicals--part II: retinal toxicity of current and new drugs. *Ophthalmic Res* 44: 205-224.

Perez-Leon J, Frech MJ, Schroder JE, Fischer F, Kneussel M, Wassle H & Backus KH. (2003) Spontaneous synaptic activity in an organotypic culture of the mouse retina. *Invest Ophthalmol Vis Sci* 44: 1376-1387.

Picard E, Fontaine I, Jonet L, Guillou F, Behar-Cohen F, Courtois Y & Jeanny JC. (2008) The protective role of transferrin in Muller glial cells after iron-induced toxicity. *Mol Vis* 14: 928-941.

Pin JP & Duvoisin R. (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* 34: 1-26.

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.

Qian DZ, Wang X, Kachhap SK, Kato Y, Wei Y, Zhang L*, et al.* (2004) The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. *Cancer Res* 64: 6626-6634.

Quigley HA. (1999) Neuronal death in glaucoma. *Prog Retin Eye Res* 18: 39-57.

Quigley HA & Addicks EM. (1981) Regional differences in the structure of the lamina cribrosa and their relation to glaucomatous optic nerve damage. *Arch Ophthalmol* 99: 137-143.

Quigley HA & Broman AT. (2006) The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol* 90: 262-267.

Quigley HA, McKinnon SJ, Zack DJ, Pease ME, Kerrigan-Baumrind LA, Kerrigan DF & Mitchell RS. (2000) Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. *Invest Ophthalmol Vis Sci* 41: 3460-3466.

Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ & Zack DJ. (1995) Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest Ophthalmol Vis Sci* 36: 774-786.

Ramakrishnan V. (1997) Histone structure and the organization of the nucleosome. *Annu Rev Biophys Biomol Struct* 26: 83-112.

Rascle A, Johnston JA & Amati B. (2003) Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5. *Mol Cell Biol* 23: 4162-4173.

Rauen T. (2000) Diversity of glutamate transporter expression and function in the mammalian retina. *Amino Acids* 19: 53-62.

Rauen T & Kanner BI. (1994) Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. *Neurosci Lett* 169: 137-140.

Reigstad LJ, Varhaug JE & Lillehaug JR. (2005) Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. *FEBS J* 272: 5723-5741.

Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M*, et al.* (2002) Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science* 295: 1077-1079.

Richmond TJ & Davey CA. (2003) The structure of DNA in the nucleosome core. *Nature* 423: 145-150.

Riepe RE & Norenburg MD. (1977) Muller cell localisation of glutamine synthetase in rat retina. *Nature* 268: 654-655.

Rodieck RW & Marshak DW. (1992) Spatial density and distribution of choline acetyltransferase immunoreactive cells in human, macaque, and baboon retinas. *J Comp Neurol* 321: 46-64.

Rohrenbeck J, Wassle H & Heizmann CW. (1987) Immunocytochemical labelling of horizontal cells in mammalian retina using antibodies against calcium-binding proteins. *Neurosci Lett* 77: 255-260.

Romano MR, Biagioni F, Besozzi G, Carrizzo A, Vecchione C, Fornai F & Lograno MD. (2012) Effects of bevacizumab on neuronal viability of retinal ganglion cells in rats. *Brain Res* 1478: 55-63.

Rosato RR & Grant S. (2005) Histone deacetylase inhibitors: insights into mechanisms of lethality. *Expert Opin Ther Targets* 9: 809-824.

Rosenberg PA. (1991) Accumulation of extracellular glutamate and neuronal death in astrocyte-poor cortical cultures exposed to glutamine. *Glia* 4: 91-100.

Rosenfeld PJ, Shapiro H, Ehrlich JS, Wong P, Marina & Groups AS. (2011) Cataract surgery in ranibizumab-treated patients with neovascular age-related macular degeneration from the phase 3 ANCHOR and MARINA trials. *Am J Ophthalmol* 152: 793-798.

Rosenstein JM, Mani N, Khaibullina A & Krum JM. (2003) Neurotrophic effects of vascular endothelial growth factor on organotypic cortical explants and primary cortical neurons. *J Neurosci* 23: 11036-11044.

Rubin L & Weisse I. (1992) Species differences relevant for ocular toxicity studies. *Manual of Oculotoxicity Testing of Drugs*: 177–191.

Ruhrberg C. (2003) Growing and shaping the vascular tree: multiple roles for VEGF. *Bioessays* 25: 1052-1060.

Ruiz-Ederra J, Garcia M, Hernandez M, Urcola H, Hernandez-Barbachano E, Araiz J & Vecino E. (2005) The pig eye as a novel model of glaucoma. *Exp Eye Res* 81: 561-569.

Russo R, Cavaliere F, Varano GP, Milanese M, Adornetto A, Nucci C*, et al.* (2013) Impairment of neuronal glutamate uptake and modulation of the glutamate transporter GLT-1 induced by retinal ischemia. *PLoS One* 8: e69250-69265.

Ryhanen T, Viiri J, Hyttinen JM, Uusitalo H, Salminen A & Kaarniranta K. (2011) Influence of Hsp90 and HDAC inhibition and tubulin acetylation on perinuclear protein aggregation in human retinal pigment epithelial cells. *J Biomed Biotechnol* 2011: 798052-798062.

Saint-Geniez M, Maharaj AS, Walshe TE, Tucker BA, Sekiyama E, Kurihara T*, et al.* (2008) Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. *PLoS One* 3: e3554-3575.

Sanchez-Armass S & Blaustein MP. (1987) Role of sodium-calcium exchange in regulation of intracellular calcium in nerve terminals. *Am J Physiol* 252: C595-603.

Sancho-Pelluz J, Alavi MV, Sahaboglu A, Kustermann S, Farinelli P, Azadi S*, et al.* (2010) Excessive HDAC activation is critical for neurodegeneration in the rd1 mouse. *Cell Death Dis* 1: e24-39.

Sancho-Pelluz J & Paquet-Durand F. (2012) HDAC inhibition prevents Rd1 mouse photoreceptor degeneration. *Adv Exp Med Biol* 723: 107-113.

Sand A, Schmidt TM & Kofuji P. (2012) Diverse types of ganglion cell photoreceptors in the mammalian retina. *Prog Retin Eye Res* 31: 287-302.

Sappington RM, Carlson BJ, Crish SD & Calkins DJ. (2010) The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest Ophthalmol Vis Sci* 51: 207-216.

Sarup V, Patil K & Sharma SC. (2004) Ciliary neurotrophic factor and its receptors are differentially expressed in the optic nerve transected adult rat retina. *Brain Res* 1013: 152-158.

Sasaoka M, Taniguchi T, Shimazawa M, Ishida N, Shimazaki A & Hara H. (2006) Intravitreal injection of endothelin-1 caused optic nerve damage following to ocular hypoperfusion in rabbits. *Exp Eye Res* 83: 629-637.

Sawaguchi K, Nakamura Y, Nakamura Y, Sakai H & Sawaguchi S. (2005) Myocilin gene expression in the trabecular meshwork of rats in a steroid-induced ocular hypertension model. *Ophthalmic Res* 37: 235-242.

Schiff LJ. (2005) Review: production, characterization, and testing of banked mammalian cell substrates used to produce biological products. *In Vitro Cell Dev Biol Anim* 41: 65-70.

Schlamp CL, Johnson EC, Li Y, Morrison JC & Nickells RW. (2001) Changes in Thy1 gene expression associated with damaged retinal ganglion cells. *Mol Vis* 7: 192-201.

Schlieve CR, Lieven CJ & Levin LA. (2006) Biochemical activity of reactive oxygen species scavengers do not predict retinal ganglion cell survival. *Invest Ophthalmol Vis Sci* 47: 3878-3886.

Schousboe A. (1981) Transport and metabolism of glutamate and GABA in neurons are glial cells. *Int Rev Neurobiol* 22: 1-45.

Schultheiss M, Januschowski K, Ruschenburg H, Schramm C, Schnichels S, Szurman P*, et al.* (2013) Dulbecco's Modified Eagle Medium is neuroprotective when compared to standard vitrectomy irrigation solution. *Graefes Arch Clin Exp Ophthalmol* 251: 1613-1619.

Schultheiss M, Schnichels S, Mlynczak T, Dipl-Ing JH, Bartz-Schmidt KU, Szurman P & Spitzer MS. (2014) Cyclosporine a protects RGC-5 cells from excitotoxic cell death. *J Glaucoma* 23: 219-224.

Segal E, Fondufe-Mittendorf Y, Chen L, Thastrom A, Field Y, Moore IK*, et al.* (2006) A genomic code for nucleosome positioning. *Nature* 442: 772-778.

Senatorov V, Malyukova I, Fariss R, Wawrousek EF, Swaminathan S, Sharan SK & Tomarev S. (2006) Expression of mutated mouse myocilin induces open-angle glaucoma in transgenic mice. *J Neurosci* 26: 11903-11914.

Senin, II, Dean KR, Zargarov AA, Akhtar M & Philippov PP. (1997) Recoverin inhibits the phosphorylation of dark-adapted rhodopsin more than it does that of

bleached rhodopsin: a possible mechanism through which rhodopsin kinase is prevented from participation in a side reaction. *Biochem J* 321: 551-555.

Seo J, Jo SA, Hwang S, Byun CJ, Lee HJ, Cho DH*, et al.* (2013) Trichostatin A epigenetically increases calpastatin expression and inhibits calpain activity and calcium-induced SH-SY5Y neuronal cell toxicity. *FEBS J* 280: 6691-6701.

Shahbazian MD & Grunstein M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* 76: 75-100.

Shareef S, Sawada A & Neufeld AH. (1999) Isoforms of nitric oxide synthase in the optic nerves of rat eyes with chronic moderately elevated intraocular pressure. *Invest Ophthalmol Vis Sci* 40: 2884-2891.

Shareef SR, Garcia-Valenzuela E, Salierno A, Walsh J & Sharma SC. (1995) Chronic ocular hypertension following episcleral venous occlusion in rats. *Exp Eye Res* 61: 379-382.

Sheppard AM, Konopka M, Robinson SR, Morgan IG & Jeffrey PL. (1991) Thy-1 antigen is specific to ganglion cells in chicks. *Neurosci Lett* 123: 87-90.

Shoshani Y, Harris A, Shoja MM, Arieli Y, Ehrlich R, Primus S*, et al.* (2012) Impaired ocular blood flow regulation in patients with open-angle glaucoma and diabetes. *Clin Experiment Ophthalmol* 40: 697-705.

Siliprandi R, Lipartiti M, Fadda E, Sautter J & Manev H. (1992) Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. *Eur J Pharmacol* 219: 173-174.

Sinn DI, Kim SJ, Chu K, Jung KH, Lee ST, Song EC*, et al.* (2007) Valproic acid-mediated neuroprotection in intracerebral hemorrhage via histone deacetylase inhibition and transcriptional activation. *Neurobiol Dis* 26: 464-472.

Sisk DR & Kuwabara T. (1985) Histologic changes in the inner retina of albino rats following intravitreal injection of monosodium L-glutamate. *Graefes Arch Clin Exp Ophthalmol* 223: 250-258.

Snell RS & Lemp MA. (1989) Clinical anatomy of the eye. *Boston: Blackwell Scientific*.

Snodderly DM, Brown PK, Delori FC & Auran JD. (1984) The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas. *Invest Ophthalmol Vis Sci* 25: 660-673.

Sommer A, Tielsch JM, Katz J, Quigley HA, Gottsch JD, Javitt J & Singh K. (1991) Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore Eye Survey. *Arch Ophthalmol* 109: 1090-1095.

Sondell M, Lundborg G & Kanje M. (1999) Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. *J Neurosci* 19: 5731-5740.

Soriano P. (1997) The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. *Development* 124: 2691-2700.

Soto I, Oglesby E, Buckingham BP, Son JL, Roberson ED, Steele MR*, et al.* (2008) Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. *J Neurosci* 28: 548-561.

Spoerri PE, Grant MB, Gomez J & Vernadakis A. (1997) Endothelial cell conditioned media mediated regulation of glutamine synthetase activity in glial cells. *Brain Res Dev Brain Res* 104: 205-208.

Stein JD, Kim DS, Niziol LM, Talwar N, Nan B, Musch DC & Richards JE. (2011) Differences in rates of glaucoma among Asian Americans and other racial groups, and among various Asian ethnic groups. *Ophthalmology* 118: 1031-1037.

Stepien KE, Rosenfeld PJ, Puliafito CA, Feuer W, Shi W, Al-Attar L*, et al.* (2009) Comparison of intravitreal bevacizumab followed by ranibizumab for the treatment of neovascular age-related macular degeneration. *Retina* 29: 1067-1073.

Stewart MW. (2011) Aflibercept (VEGF-TRAP): the next anti-VEGF drug. *Inflamm Allergy Drug Targets* 10: 497-508.

Stoilov I, Akarsu AN & Sarfarazi M. (1997) Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary

congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. *Hum Mol Genet* 6: 641-647.

Stokely ME, Brady ST & Yorio T. (2002) Effects of endothelin-1 on components of anterograde axonal transport in optic nerve. *Invest Ophthalmol Vis Sci* 43: 3223-3230.

Stramm LE, Haskins ME, McGovern MM & Aguirre GD. (1983) Tissue culture of cat retinal pigment epithelium. *Exp Eye Res* 36: 91-101.

Strauss O. (2005) The retinal pigment epithelium in visual function. *Physiol Rev* 85: 845-881.

Sullivan RK, Woldemussie E, Macnab L, Ruiz G & Pow DV. (2006) Evoked expression of the glutamate transporter GLT-1c in retinal ganglion cells in human glaucoma and in a rat model. *Invest Ophthalmol Vis Sci* 47: 3853-3859.

Sumbul U, Song S, McCulloch K, Becker M, Lin B, Sanes JR*, et al.* (2014) A genetic and computational approach to structurally classify neuronal types. *Nat Commun* 5: 3512.

Suzuki S, Yamashita T, Tanaka K, Hattori H, Sawamoto K, Okano H & Suzuki N. (2005) Activation of cytokine signaling through leukemia inhibitory factor receptor (LIFR)/gp130 attenuates ischemic brain injury in rats. *J Cereb Blood Flow Metab* 25: 685-693.

Svensson B, Peters M, Konig HG, Poppe M, Levkau B, Rothermundt M*, et al.* (2002) Vascular endothelial growth factor protects cultured rat hippocampal neurons against hypoxic injury via an antiexcitotoxic, caspase-independent mechanism. *J Cereb Blood Flow Metab* 22: 1170-1175.

Tagawa K, Marubuchi S, Qi ML, Enokido Y, Tamura T, Inagaki R*, et al.* (2007) The induction levels of heat shock protein 70 differentiate the vulnerabilities to mutant huntingtin among neuronal subtypes. *J Neurosci* 27: 868-880.

Takahashi K, Saishin Y, Saishin Y, King AG, Levin R & Campochiaro PA. (2009) Suppression and regression of choroidal neovascularization by the multitargeted kinase inhibitor pazopanib. *Arch Ophthalmol* 127: 494-499.

Takayama S, Reed JC & Homma S. (2003) Heat-shock proteins as regulators of apoptosis. *Oncogene* 22: 9041-9047.

Tammela T, Petrova TV & Alitalo K. (2005) Molecular lymphangiogenesis: new players. *Trends Cell Biol* 15: 434-441.

Tang J, Yan H & Zhuang S. (2013) Histone deacetylases as targets for treatment of multiple diseases. *Clin Sci (Lond)* 124: 651-662.

Tang Z, Arjunan P, Lee C, Li Y, Kumar A, Hou X*, et al.* (2010) Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3beta phosphorylation. *J Exp Med* 207: 867-880.

Tatton NA, Tezel G, Insolia SA, Nandor SA, Edward PD & Wax MB. (2001) In situ detection of apoptosis in normal pressure glaucoma. a preliminary examination. *Surv Ophthalmol* 45 Suppl 3: S268-272; discussion S273-266.

Tektas OY, Hammer CM, Danias J, Candia O, Gerometta R, Podos SM & Lutjen-Drecoll E. (2010) Morphologic changes in the outflow pathways of bovine eyes treated with corticosteroids. *Invest Ophthalmol Vis Sci* 51: 4060-4066.

Tezel G, Hernandez R & Wax MB. (2000) Immunostaining of heat shock proteins in the retina and optic nerve head of normal and glaucomatous eyes. *Arch Ophthalmol* 118: 511-518.

Tezel G, Kass MA, Kolker AE, Becker B & Wax MB. (1997) Plasma and aqueous humor endothelin levels in primary open-angle glaucoma. *J Glaucoma* 6: 83-89.

Tezel G, Li LY, Patil RV & Wax MB. (2001) TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes. *Invest Ophthalmol Vis Sci* 42: 1787-1794.

Thanos S & Thiel HJ. (1990) Regenerative and proliferative capacity of adult human retinal cells in vitro. *Graefes Arch Clin Exp Ophthalmol* 228: 369-376.

Tielsch JM, Katz J, Sommer A, Quigley HA & Javitt JC. (1995) Hypertension, perfusion pressure, and primary open-angle glaucoma. A population-based assessment. *Arch Ophthalmol* 113: 216-221.

Tielsch JM, Sommer A, Katz J, Royall RM, Quigley HA & Javitt J. (1991) Racial variations in the prevalence of primary open-angle glaucoma. The Baltimore Eye Survey. *JAMA* 266: 369-374.

Tirosh I & Barkai N. (2008) Two strategies for gene regulation by promoter nucleosomes. *Genome Res* 18: 1084-1091.

Toops KA, Berlinicke C, Zack DJ & Nickells RW. (2012) Hydrocortisone stimulates neurite outgrowth from mouse retinal explants by modulating macroglial activity. *Invest Ophthalmol Vis Sci* 53: 2046-2061.

Toris CB, Camras CB & Yablonski ME. (1999) Acute versus chronic effects of brimonidine on aqueous humor dynamics in ocular hypertensive patients. *Am J Ophthalmol* 128: 8-14.

Trivino A, Ramirez JM, Ramirez AI, Salazar JJ & Garcia-Sanchez J. (1997) Comparative study of astrocytes in human and rabbit retinae. *Vision Res* 37: 1707-1711.

Tsacopoulos M & Magistretti PJ. (1996) Metabolic coupling between glia and neurons. *J Neurosci* 16: 877-885.

Vakulenko SB & Mobashery S. (2003) Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* 16: 430-450.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M & Telser J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 39: 44-84.

Valko M, Morris H & Cronin MT. (2005) Metals, toxicity and oxidative stress. *Curr Med Chem* 12: 1161-1208.

Van Bergen NJ, Wood JP, Chidlow G, Trounce IA, Casson RJ, Ju WK*, et al.* (2009) Recharacterization of the RGC-5 retinal ganglion cell line. *Invest Ophthalmol Vis Sci* 50: 4267-4272.

Van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, Knudsen LE*, et al.* (2010) Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro* 24: 1053-1063.

Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G*, et al.* (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro* 18: 1-12.

Vaquero A, Loyola A & Reinberg D. (2003) The constantly changing face of chromatin. *Sci Aging Knowledge Environ* 2003: 4-20.

Veth KN, Willer JR, Collery RF, Gray MP, Willer GB, Wagner DS*, et al.* (2011) Mutations in zebrafish lrp2 result in adult-onset ocular pathogenesis that models myopia and other risk factors for glaucoma. *PLoS Genet* 7: e1001310-1001323.

Voigt T. (1986) Cholinergic amacrine cells in the rat retina. *J Comp Neurol* 248: 19-35.

Von Toerne C, Menzler J, Ly A, Senninger N, Ueffing M & Hauck SM. (2014) Identification of a novel neurotrophic factor from primary retinal Muller cells using SILAC. *Mol Cell Proteomics* 13: 2371-2381.

Vorwerk CK, Lipton SA, Zurakowski D, Hyman BT, Sabel BA & Dreyer EB. (1996) Chronic low-dose glutamate is toxic to retinal ganglion cells. Toxicity blocked by memantine. *Invest Ophthalmol Vis Sci* 37: 1618-1624.

Vrana JA, Decker RH, Johnson CR, Wang Z, Jarvis WD, Richon VM*, et al.* (1999) Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. *Oncogene* 18: 7016-7025.

Wakabayashi K, Nagai A, Sheikh AM, Shiota Y, Narantuya D, Watanabe T*, et al.* (2010) Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J Neurosci Res* 88: 1017-1025.

Walkinshaw DR, Tahmasebi S, Bertos NR & Yang XJ. (2008) Histone deacetylases as transducers and targets of nuclear signaling. *J Cell Biochem* 104: 1541-1552.

Wang C, Fu M, Mani S, Wadler S, Senderowicz AM & Pestell RG. (2001) Histone acetylation and the cell-cycle in cancer. *Front Biosci* 6: D610-629.

Wang DY, Ray A, Rodgers K, Ergorul C, Hyman BT, Huang W & Grosskreutz CL. (2010a) Global gene expression changes in rat retinal ganglion cells in experimental glaucoma. *Invest Ophthalmol Vis Sci* 51: 4084-4095.

Wang SW, Mu X, Bowers WJ & Klein WH. (2002) Retinal ganglion cell differentiation in cultured mouse retinal explants. *Methods* 28: 448-456.

Wang X, Harmon J, Zabrieskie N, Chen Y, Grob S, Williams B*, et al.* (2010b) Using the Utah Population Database to assess familial risk of primary open angle glaucoma. *Vision Res* 50: 2391-2395.

Wang X, Ng YK & Tay SS. (2005) Factors contributing to neuronal degeneration in retinas of experimental glaucomatous rats. *J Neurosci Res* 82: 674-689.

Wang Y, Kudoh J, Kubota R, Asakawa S, Minoshima S & Shimizu N. (1996) Chromosomal mapping of a family of human glutamine synthetase genes: functional gene (GLUL) on 1q25, pseudogene (GLULP) on 9p13, and three related genes (GLULL1, GLULL2, GLULL3) on 5q33, 11p15, and 11q24. *Genomics* 37: 195-199.

Waniewski RA & Martin DL. (1986) Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. *J Neurochem* 47: 304-313.

Ware CB, Horowitz MC, Renshaw BR, Hunt JS, Liggitt D, Koblar SA*, et al.* (1995) Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* 121: 1283-1299.

Wassle H, Grunert U, Chun MH & Boycott BB. (1995) The rod pathway of the macaque monkey retina: identification of AII-amacrine cells with antibodies against calretinin. *J Comp Neurol* 361: 537-551.

Wassle H, Grunert U & Rohrenbeck J. (1993) Immunocytochemical staining of AII-amacrine cells in the rat retina with antibodies against parvalbumin. *J Comp Neurol* 332: 407-420.

Watanabe M & Rodieck RW. (1989) Parasol and midget ganglion cells of the primate retina. *J Comp Neurol* 289: 434-454.

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: 733-742.

Watanabe T. (2010) Investigational histone deacetylase inhibitors for non-Hodgkin lymphomas. *Expert Opin Investig Drugs* 19: 1113-1127.

Wax MB, Tezel G, Yang J, Peng G, Patil RV, Agarwal N*, et al.* (2008) Induced autoimmunity to heat shock proteins elicits glaucomatous loss of retinal

ganglion cell neurons via activated T-cell-derived fas-ligand. *J Neurosci* 28: 12085-12096.

Webber CA, Chen YY, Hehr CL, Johnston J & McFarlane S. (2005) Multiple signaling pathways regulate FGF-2-induced retinal ganglion cell neurite extension and growth cone guidance. *Mol Cell Neurosci* 30: 37-47.

Weber AJ & Zelenak D. (2001) Experimental glaucoma in the primate induced by latex microspheres. *J Neurosci Methods* 111: 39-48.

Wei L, Fraser JL, Lu ZY, Hu X & Yu SP. (2012) Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiol Dis* 46: 635-645.

Weichert W, Roske A, Gekeler V, Beckers T, Ebert MP, Pross M*, et al.* (2008) Association of patterns of class I histone deacetylase expression with patient prognosis in gastric cancer: a retrospective analysis. *Lancet Oncol* 9: 139-148.

Weinreb RN & Harris A. (2009) World Glaucoma Association Consensus Series – 6. Ocular Blood Flow in Glaucoma. *Amsterdam:Kugler Publications*.

Weinreb RN & Lindsey JD. (2005) The importance of models in glaucoma research. *J Glaucoma* 14: 302-304.

Weishaupt JH, Klocker N & Bahr M. (2005) Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells. *J Mol Neurosci* 26: 17-25.

Wender R, Brown AM, Fern R, Swanson RA, Farrell K & Ransom BR. (2000) Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J Neurosci* 20: 6804-6810.

Wessman SJ & Levings RL. (1999) Benefits and risks due to animal serum used in cell culture production. *Dev Biol Stand* 99: 3-8.

Whigham HM, Brooks DE, Andrew SE, Gelatt KN, Strubbe DT & Biros DJ. (1999) Treatment of equine glaucoma by transscleral neodymium:yttrium aluminum garnet laser cyclophotocoagulation: a retrospective study of 23 eyes of 16 horses. *Vet Ophthalmol* 2: 243-250.

Whitney MJ, Lee A, Ylostalo J, Zeitouni S, Tucker A & Gregory CA. (2009) Leukemia inhibitory factor secretion is a predictor and indicator of early progenitor status in adult bone marrow stromal cells. *Tissue Eng Part A* 15: 33-44.

Wiechmann AF, Akots G, Hammarback JA, Pettenati MJ, Rao PN & Bowden DW. (1994) Genetic and physical mapping of human recoverin: a gene expressed in retinal photoreceptors. *Invest Ophthalmol Vis Sci* 35: 325-331.

Wiechmann AF & Hammarback JA. (1993) Expression of recoverin mRNA in the human retina: localization by in situ hybridization. *Exp Eye Res* 57: 763-769.

Wilfert JN, Burke JP, Bloomer HA & Smith CB. (1971) Renal insufficiency associated with gentamicin therapy. *J Infect Dis* 124 Suppl: S148-155.

Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP*, et al.* (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336: 684-687.

Wilson AJ, Byun DS, Popova N, Murray LB, L'Italien K, Sowa Y*, et al.* (2006) Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* 281: 13548-13558.

Winzeler A & Wang JT. (2013) Purification and culture of retinal ganglion cells from rodents. *Cold Spring Harb Protoc* 2013: 643-652.

Witt O, Deubzer HE, Milde T & Oehme I. (2009) HDAC family: What are the cancer relevant targets? *Cancer Lett* 277: 8-21.

Woldemussie E, Wijono M & Pow D. (2007) Localization of alpha 2 receptors in ocular tissues. *Vis Neurosci* 24: 745-756.

Woldemussie E, Wijono M & Ruiz G. (2004) Muller cell response to laser-induced increase in intraocular pressure in rats. *Glia* 47: 109-119.

Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiestler OD & Blumcke I. (1996) NeuN: a useful neuronal marker for diagnostic histopathology. *J Histochem Cytochem* 44: 1167-1171.

Wolfs RC, Klaver CC, Ramrattan RS, van Duijn CM, Hofman A & de Jong PT. (1998) Genetic risk of primary open-angle glaucoma. Population-based familial aggregation study. *Arch Ophthalmol* 116: 1640-1645.

Wood JP, DeSantis L, Chao HM & Osborne NN. (2001) Topically applied betaxolol attenuates ischaemia-induced effects to the rat retina and stimulates BDNF mRNA. *Exp Eye Res* 72: 79-86.

Wood JP, Schmidt KG, Melena J, Chidlow G, Allmeier H & Osborne NN. (2003) The beta-adrenoceptor antagonists metipranolol and timolol are retinal neuroprotectants: comparison with betaxolol. *Exp Eye Res* 76: 505-516.

Wu VW, Nishiyama N & Schwartz JP. (1998) A culture model of reactive astrocytes: increased nerve growth factor synthesis and reexpression of cytokine responsiveness. *J Neurochem* 71: 749-756.

Wu X, Chen PS, Dallas S, Wilson B, Block ML, Wang CC*, et al.* (2008) Histone deacetylase inhibitors up-regulate astrocyte GDNF and BDNF gene transcription and protect dopaminergic neurons. *Int J Neuropsychopharmacol* 11: 1123-1134.

Xiao W, Chen X, Liu X, Luo L, Ye S & Liu Y. (2014) Trichostatin A, a histone deacetylase inhibitor, suppresses proliferation and epithelial-mesenchymal transition in retinal pigment epithelium cells. *J Cell Mol Med* 18: 646-655.

Xin H, Yannazzo JA, Duncan RS, Gregg EV, Singh M & Koulen P. (2007) A novel organotypic culture model of the postnatal mouse retina allows the study of glutamate-mediated excitotoxicity. *J Neurosci Methods* 159: 35-42.

Yamamori T, Fukada K, Aebersold R, Korsching S, Fann MJ & Patterson PH. (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* 246: 1412-1416.

Yang Z, Quigley HA, Pease ME, Yang Y, Qian J, Valenta D & Zack DJ. (2007) Changes in gene expression in experimental glaucoma and optic nerve transection: the equilibrium between protective and detrimental mechanisms. *Invest Ophthalmol Vis Sci* 48: 5539-5548.

Yoneda S, Tanihara H, Kido N, Honda Y, Goto W, Hara H & Miyawaki N. (2001) Interleukin-1beta mediates ischemic injury in the rat retina. *Exp Eye Res* 73: 661-667.

Yoshida M, Kijima M, Akita M & Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol Chem* 265: 17174-17179.

Yu K, Ge J, Summers JB, Li F, Liu X, Ma P*, et al.* (2008) TSP-1 secreted by bone marrow stromal cells contributes to retinal ganglion cell neurite outgrowth and survival. *PLoS One* 3: e2470-2481.

Yu S, Tanabe T, Dezawa M, Ishikawa H & Yoshimura N. (2006a) Effects of bone marrow stromal cell injection in an experimental glaucoma model. *Biochem Biophys Res Commun* 344: 1071-1079.

Yu S, Tanabe T & Yoshimura N. (2006b) A rat model of glaucoma induced by episcleral vein ligation. *Exp Eye Res* 83: 758-770.

Zhang L, Jin S, Wang C, Jiang R & Wan J. (2010a) Histone deacetylase inhibitors attenuate acute lung injury during cecal ligation and puncture-induced polymicrobial sepsis. *World J Surg* 34: 1676-1683.

Zhang M, Marshall B & Atherton SS. (2008) Murine cytomegalovirus infection and apoptosis in organotypic retinal cultures. *Invest Ophthalmol Vis Sci* 49: 295-303.

Zhang XM, Li Liu DT, Chiang SW, Choy KW, Pang CP, Lam DS & Yam GH. (2010b) Immunopanning purification and long-term culture of human retinal ganglion cells. *Mol Vis* 16: 2867-2872.

Zhang Y, Cho CH, Atchaneeyasakul LO, McFarland T, Appukuttan B & Stout JT. (2005) Activation of the mitochondrial apoptotic pathway in a rat model of central retinal artery occlusion. *Invest Ophthalmol Vis Sci* 46: 2133-2139.

Zhang Y, Li N, Caron C, Matthias G, Hess D, Khochbin S & Matthias P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J* 22: 1168-1179.

Zhang Z, Qin X, Tong N, Zhao X, Gong Y, Shi Y & Wu X. (2012) Valproic acid-mediated neuroprotection in retinal ischemia injury via histone deacetylase inhibition and transcriptional activation. *Exp Eye Res* 94: 98-108.

Zhao T, Li Y, Tang L, Li Y, Fan F & Jiang B. (2011) Protective effects of human umbilical cord blood stem cell intravitreal transplantation against optic nerve injury in rats. *Graefes Arch Clin Exp Ophthalmol* 249: 1021-1028.

Zheng J, Feng X, Hou L, Cui Y, Zhu L, Ma J*, et al.* (2011) Latanoprost promotes neurite outgrowth in differentiated RGC-5 cells via the PI3K-Akt-mTOR signaling pathway. *Cell Mol Neurobiol* 31: 597-604.

Zheng L, Yan Y, An J, Zhang L, Liu W, Xia F & Zhang Z. (2012) Retinal horizontal cells reduced in a rat model of congenital stationary night blindness. *Neurosci Lett* 521: 26-30.

Zwart I, Hill AJ, Al-Allaf F, Shah M, Girdlestone J, Sanusi AB*, et al.* (2009) Umbilical cord blood mesenchymal stromal cells are neuroprotective and promote regeneration in a rat optic tract model. *Exp Neurol* 216: 439-448.

Experimental Eye Research 143 (2016) 28–38

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Human organotypic retinal cultures (HORCs) as a chronic experimental model for investigation of retinal ganglion cell degeneration

Andrew Osborne [a, b], Marina Hopes [a, c], Phillip Wright [a], David C. Broadway [a, c, d], Julie Sanderson [a, *]

[a] School of Pharmacy, University of East Anglia, Norwich, NR4 7TJ, UK
[b] John van Geest Centre for Brain Repair, University of Cambridge, Cambridge, CB2 0PY, UK
[c] Department of Ophthalmology, Norfolk and Norwich University Hospital, Norwich, NR4 7UY, UK
[d] School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK

ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form
18 September 2015
Accepted in revised form 22 September 2015
Available online 1 October 2015

Keywords:
Human
RGC
Glaucoma
Model
Retina
Apoptosis
Degeneration

ABSTRACT

There is a growing need for models of human diseases that utilise native, donated human tissue in order to model disease processes and develop novel therapeutic strategies. In this paper we assessed the suitability of adult human retinal explants as a potential model of chronic retinal ganglion cell (RGC) degeneration. Our results confirmed that RGC markers commonly used in rodent studies (NeuN, βIII Tubulin and Thy-1) were appropriate for labelling human RGCs and followed the expected differential expression patterns across, as well as throughout, the macular and para-macular regions of the retina. Furthermore, we showed that neither donor age nor *post-mortem* time (within 24 h) significantly affected the initial expression levels of RGC markers. In addition, the feasibility of using human *post mortem* donor tissue as a long-term model of RGC degeneration was determined with RGC protein being detectable up to 4 weeks in culture with an associated decline in RGC mRNA and significant, progressive, apoptotic labelling of $NeuN^+$ cells. Differences in RGC apoptosis might have been influenced by medium compositions indicating that media constituents could play a role in supporting axotomised RGCs. We propose that using *ex vivo* human explants may prove to be a useful model for testing the effectiveness of neuroprotective strategies.

1. Introduction

Organotypic retinal cultures provide an important link between dissociated cell cultures and *in vivo* models (Johnson et al., 2011), benefiting from maintenance of heterogeneous cell populations that can be observed *in situ* (Caffé et al., 2001). Although cultures of dissociated cells are helpful in elucidating the direct effect of drugs on individual cell types, the loss of intercellular relationships limits their usefulness when modelling complex diseases (Buyens et al., 2014). At the other end of the spectrum, animal models that more closely resemble actual pathologies are costly, time consuming and the outcomes can be difficult to correlate with human conditions. Furthermore, there is a recognized demand for *in vitro* models that can replace or reduce the need for animal experiments.

Although research using retinal explants has increased over recent years, the use of human retinal explants is still underutilised. Research using rodent retinal organotypic cultures is now common but it is recognised that differences exist between rodent and human retina (Albrecht May, 2008; Bobu et al., 2008; Levkovitch-Verbin, 2004). The most significant difference between mouse and human retina is that the human retina has a central macula and fovea for high-resolution colour vision, in addition to different ratios of cone and rod photoreceptors with specific topography and subtypes of retinal ganglion cells (RGCs). Using human retina therefore, provides the most appropriate tissue to model a variety of human eye diseases.

Some of the factors limiting the advancement of human retinal models are that questions remain to be answered regarding donor

Abbreviations: HORCs, human organotypic retinal cultures; RGCs, retinal ganglion cells.

* Corresponding author.
E-mail address: j.sanderson@uea.ac.uk (J. Sanderson).

http://dx.doi.org/10.1016/j.exer.2015.09.012
0014-4835/

variability, the effects of *post mortem* time and the potential time scale that the human retina can survive in culture. There are also practical issues to consider, such as whether markers used in animal experiments are appropriate for use in human research. The present paper aimed to investigate these questions.

Firstly, the expression and labelling of numerous RGC markers was investigated across the human retina within the macular and para-macular regions. In addition, the variation of RGC mRNA expression was evaluated from numerous *post mortem* donor eyes to address concerns relating to donor variability. Further work to address possible effects of *post mortem* time on tissue viability were assessed through the comparison of retina derived immediately after orbital exenteration from living donors and those from eyes obtained at varying times after donor patient death. Secondly, we aimed to assess the feasibility of using adult human retina in a long-term culture system for studying RGC degeneration. We have previously shown that adult human organotypic retinal cultures (HORCs) can be used to investigate RGC death following brief ischaemic injury (Niyadurupola et al., 2013; Osborne et al., 2015) and glutamate excitotoxicity (Niyadurupola et al., 2011), but degeneration over time points longer than 48 h had not been investigated. Human embryonic (Engelsberg et al., 2008) and foetal (Donovan and Dyer, 2006; Rojas et al., 2003; Zhang et al., 2010) retinal explants have been cultured for up to 42 days with preservation of tissue cytoarchitecture and most intraretinal connections. Furthermore, various retinal cell markers are still observable in adult human explants after 9 days in culture (Fernandez-Bueno et al., 2012). However, RGC degeneration over prolonged periods has not been investigated in an adult human system.

Understanding ways to model and possibly delay the onset of RGC apoptosis will play an important role in limiting the effects of optic nerve atrophy and aid in the advancement of future regenerative strategies to promote axon regrowth that require the preservation of RGCs after injury.

2. Materials and methods

2.1. Human explant dissection and culture

Donor human eyes were obtained within 24 h *post mortem* from the East Anglian Eye Bank or within 2 h from patients undergoing orbital exenteration under the auspices of the Human Tissue Bank (both facilities within the Norfolk and Norwich University Hospital). All donated material was free of any known retinal pathology and contained no obvious ocular trauma or undiagnosed retinal injury at dissection. Fifty-two *post mortem* donor eyes were used in this study between the age of 30 and 91 years. Unpublished data from a larger cohort of untreated eyes (n = 100 donor eyes) was also included for comparative purposes. The four living donor eyes were kindly donated from patients between 59 and 84 years of age. The research was approved by the UK National Research Ethics Committee (REC 04/Q0102/57) and was conducted under the tenets of the Declaration of Helsinki.

Retinal dissection was performed as described previously (Niyadurupola et al., 2011). Briefly, the anterior portion (including the lens and iris) of each donor eye was removed carefully by a circumferential incision at the pars plana (Fig. 1A). A flat retinal preparation was established by removal of the vitreous and cutting small incisions in the peripheral retina (Fig. 1D). The macula removed using a 4 mm diameter dissecting trephine (Biomedical Research Instruments, MD, USA) (Fig. 1E) and five para-macular explants, termed human organotypic retinal cultures (HORCs), were dissected from each donor retina using the same trephine. The location of each retinal HORC explant was equidistant from the macula for each donor retina using a template to reduce variability between dissections (Fig. 1G).

4 mm circular explants were randomised prior to culture for up to 4 weeks in 35 mm culture dishes (Corning, NY, USA) containing 1.5 ml of freshly prepared medium. Culture medium included 1) serum free (SF) Dulbecco's Modified Eagle Medium (DMEM)/HamF12, 2) 10% foetal bovine serum (10% FBS) supplemented DMEM/HamF12 or 3) Neurobasal®-A media (NB) with 2% B27 supplement, 1% N2 supplement and 0.8 mM L-glutamine (All from Invitrogen, Paisley, UK). All media were supplemented with gentamicin (50 μg/ml; Sigma–Aldrich, Poole, UK) and explants were cultured at 35 °C in a humidified atmosphere of 95% air/5% CO_2. Explants were cultured as free-floating preparations, immersed in medium, the RGC side facing upwards. Media was replaced immediately after dissection and half the medium was exchanged twice weekly thereafter.

2.2. Planar retinal sectioning

4 mm retinal explants were taken from the macular and para-macular retina of 4 donor eyes, placed RGC side up on filter paper and mounted on a prepared surface of frozen optimal cutting temperature compound (OCT) (Sakura Finetek, Zoeterwoude, Netherlands) as described previously (Niyadurupola et al., 2013). Further OCT was used to cover the sample prior to freezing. 20 μm sections in the plane of the retinal nuclear layers were taken using a Bright OTF 5000 cryostat (Bright Instruments, Huntingdon, UK) and individually collected in 1.5 ml eppendorfs before being frozen on dry ice.

2.3. Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from 221 HORCs using the RNeasy Mini Kit (Qiagen, Crawley, UK) or from 107 planar retinal sections using the RNeasy Micro kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and reverse transcribed to complementary DNA (cDNA) in a reaction catalysed by Superscript™ II reverse transcriptase with dNTP mix and random primers (all Invitrogen, Paisley, UK).

TaqMan PCR was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) using 5 ng of input cDNA, Mastermix (Applied Biosystems, Warrington, UK) and probes/primers listed in Table 1. Whole explant mRNA expression was normalised to the geometric mean of CT values for cytochrome c-1 (*CYC1*) and topoisomerase DNA I (*TOP1*) as described previously (Niyadurupola et al., 2011), whilst planar sections were normalised to the section containing the greatest level of expression. Expression profiles from individual retinas were aligned by matching expression of the photoreceptor marker recoverin (*RCVRN*).

2.4. Immunohistochemistry and TUNEL analysis

Immunohistochemistry was used to localise and quantify RGC number. 65 explants were fixed in 4% formaldehyde for 24 h and then cryopreserved in a 30% sucrose solution in PBS at 4 °C for a further 24 h. Explants were embedded in OCT and frozen at −80 °C. Transverse 13 μm sections were cut via cryostat and mounted on 3'aminopropyl-triethoxyl silane (TESPA, Sigma–Aldrich, Poole, UK) coated glass slides.

Sections were immunostained overnight with RGC markers neuronal nuclei (NeuN) (mouse; 1:250, Millipore, Watford, UK), βIII tubulin (mouse; 1:1000, G7121 Promega, Southampton, UK or

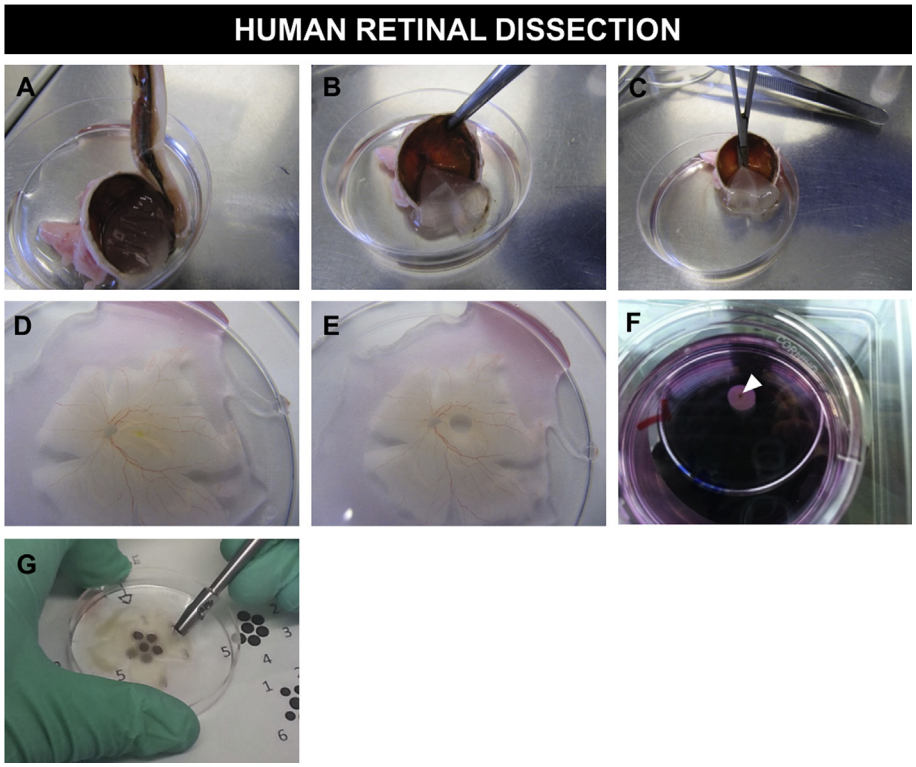


Fig. 1. Dissection of the human eye globe to create retinal explants. (A) A circular ring of tissue was removed approximately 10 mm below the ciliary body. (B) The eye globe was rotated to dissociate the retina from the underlying retinal pigmented epithelium using the weight of the vitreous to separate the layers. (C) A single cut was performed at the optic nerve head to detach the retina. (D) The vitreous was then removed and the retina spread flat, retinal ganglion cell side up. (E) The macula was removed using a 4 mm diameter dissecting trephine. The fovea (white arrow) at the centre of the macula could be identified as a shallow depression containing yellow carotenoid pigments lutein and zeaxanthin that indicated accurate macula explant preparation (F). (G) Five 4 mm para-macular samples were taken at equidistant locations from the macula using a template to reduce variability between dissections. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rabbit; 1:1000 MRB-435 Covance, Maidenhead, UK) or Thy-1.1 (mouse; 1:100, Millipore, Watford, UK) diluted in blocking solution (5% goat serum (Invitrogen, Paisley, UK), 0.3% Triton X-100 (Sigma–Aldrich, Poole, UK) in PBS). The DeadEnd™ Fluorometric TUNEL system (TUNEL) (G3250 Promega, Southampton, UK) was performed alongside NeuN immunohistochemistry to visualise apoptotic RGCs as performed previously (Niyadurupola et al., 2011; Osborne et al., 2015). TUNEL staining was carried out the following morning after overnight primary antibody incubation according to manufacturer's instructions. Secondary antibodies of Alexa Fluor 488- and 568–conjugated (Invitrogen, Paisley, UK) (1:1000) were used diluted in blocking solution for 2 h and nuclei were counterstained with DAPI (1:100; Invitrogen, Paisley, UK).

10 whole-mount explants were fixed in 4% paraformaldehyde for 24 h followed by 5x PBS washes and blocking (5% goat serum, 0.2% bovine serum albumin (Sigma–Aldrich, Poole, UK), 0.3% Triton X-100 in PBS) for 1 h. Co-localisation between (NeuN) (1:200 Millipore) and βIII tubulin (1:1000 Covance) was performed via overnight sequential staining using antibodies raised in different species. Following washing and secondary antibody staining, whole-mounts were placed on slides, RGC side up, between two thin plastic parafilm strips (Sigma–Aldrich, Poole, UK) running along either edge of the slide to act as spacers. Samples were then mounted with fluorSave™ reagent (Calbiochem/EMD Chemicals Inc., Gibbstown, NJ, USA) and a coverslip placed on top.

Images were taken using a wide-field Axiovert 200M fluorescence microscope (Zeiss, Welwyn Garden City, UK) or SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) at 20 or 40X magnification. Confocal images were obtained via sequential scanning using 0.5 μm z-step intervals.

The number of DAPI-labelled nuclei in the RGC layer was quantified using the ImageJ plugin 'Image-based Tool for Counting Nuclei (ITCN)' (http://www.bioimage.ucsb.edu/downloads/automatic-nuclei-counter-plug-in-for-imagej). Software accuracy was >95% comparable to manual counts and any missed nuclei were added using the 'Cell Counter' Plugin. RGC counting and the number of TUNEL^{+} NeuN labelled cells were assessed manually by a masked investigator from 20 non-overlapping regions per explant. Co-localisation was assessed using Velocity software with an acceptance reading of >0.9 between the two fluorescent dyes. The images shown were from a stack depth of 3 μm where clear distinction between individual RGCs could be observed.

Table 1
List of TaqMan probe/primer sets used in the experiments.

Gene	Number/sequence	Supplier
RBFOX3	Hs01370653_m1	Applied Biosystems, Warrington, UK
TUB3	Hs00801390_s1	Applied Biosystems, Warrington, UK
THY1	Hs00174816_m1	Applied Biosystems, Warrington, UK
TOP1	HK-DD-hu-300	Primer Design, Southampton, UK
CYC1	HK-DD-hu-300	Primer Design, Southampton, UK

2.5. Statistical analysis

Data shown is the mean ± standard error of the mean (s.e.m). Significance was determined using Student's t-tests or one-way ANOVA with Dunnett's or Tukey's post hoc test (GraphPad Prism; Graph-Pad Software Inc., La Jolla, Ca, USA). Differences were considered significant at the $p \leq 0.05$ level.

3. Results

3.1. RGC marker gene expression in retinal explants

As an indicator of RGC number, the mRNA expression pattern of three commonly used RGC markers (*RBFOX3*, *TUB3* and *THY1*) in human retinal explants (Fig. 2) was investigated. Comparable levels of RGC gene expression were seen in each of the five para-macular explants (no significant differences between explants were observed based on their sampling distribution, *RBFOX3* $p = 0.8480$, *TUB3* $p = 0.9761$, *THY1* $p = 0.9196$, Fig. 2B, C, D). The macula (M), as expected, showed the greatest expression of each marker.

To address concerns relating to donor variability with respect to *post mortem* time, we compared RGC gene expression from *post mortem* samples to that of explants from living donors, when retinal tissue had been obtained as quickly as possible (<2 h) following orbital exenteration. Results indicated no significant differences in retinal *RBFOX3*, *TUB3* and *THY1* expression between the living donor samples and those excised from *post mortem* tissue (Fig. 2 – red dots).

Further comparisons to assess RGC variations with age were carried out by plotting individual explant gene expression data points against age, which yielded no significant correlation (Fig. 3A, C, E). A similar lack of correlation was observed when comparing *RBFOX3*, *TUB3* and *THY1* to *post mortem* time (Fig. 3B, D, F). Addressing these issues further by comparing *THY1* expression from explants collected from 100 *post mortem* donor eyes revealed the same outcome that neither age ($p = 0.0911$, Fig. 3G) nor *post mortem* time ($p = 0.8251$, Fig. 3H) had any significant influence on RGC expression within the human retina.

3.2. RGC mRNA expression profiling in para-macular and macula explants

To confirm that the investigated RGC markers were expressed in the correct region of the retina, planar sectioning was used. *RBFOX3*, *TUB3* and *THY1* each showed highest expression in the inner retina for both macular and para-macular explants (Fig. 4). As expected, the macula explant yielded a larger number of sections (approximately 20 compared to 10 at the para-macular locations) due to greater retinal thickness (Fig. 4A). Of the macular sections, a larger number contained RGC mRNA (approximately 8 compared to 4 at the para-macular locations) resulting in a broader peak compared to para-macular regions (Fig. 4B). A similar expression pattern was seen between each of the three RGC markers, which indicated that the markers were likely to be recognising the same cell type.

3.3. Comparison of RGC protein markers in retinal explants

Having shown that *RBFOX3*, *TUB3* and *THY1* represented useful markers for human RGCs, being differentially expressed across and throughout the retina in their expected locations, we wished to support this data via observation of their protein expression.

NeuN, βIII tubulin and Thy-1 immunohistochemistry in both macular and para-macular human retinal explants supported gene

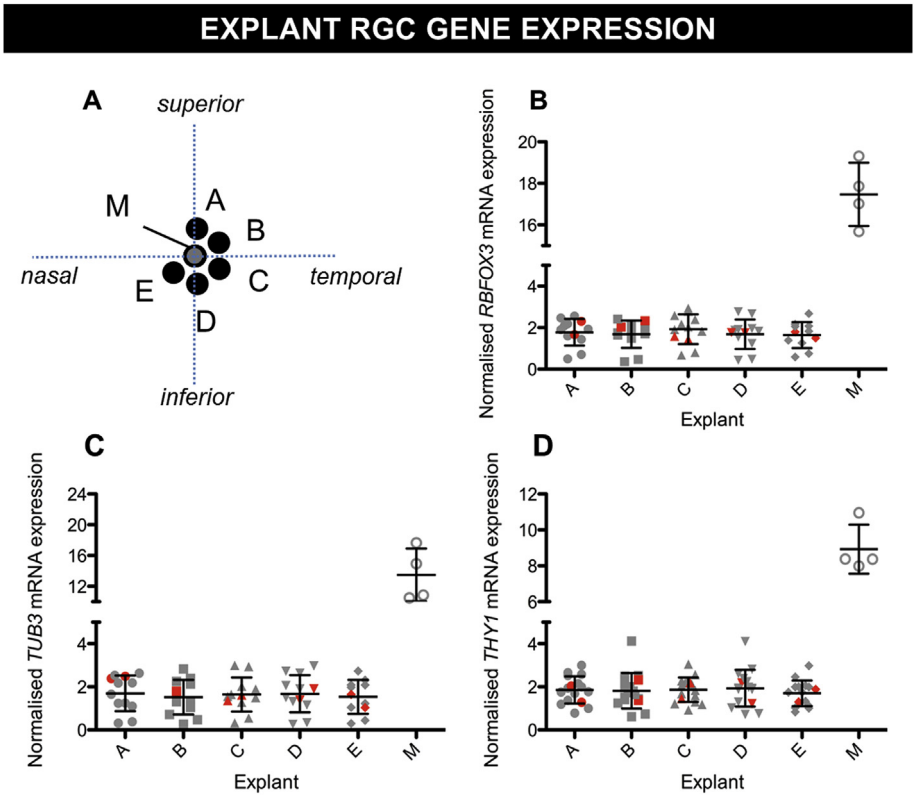


Fig. 2. RGC gene expression was comparable between explants taken in the para-macular region and the greatest expression of each marker was within macular explants. (A) The dissection template used to collect all explants. (B) *RBFOX3* (NeuN) mRNA expression in retinal explants (n = 11 donor eyes). (C) *TUB3* (βIII tubulin) mRNA expression in explants (n = 11 donor eyes). (D) *THY1* mRNA expression in explants (n = 15 donor eyes). All mRNA was normalised to the expression of the housekeeping genes *TOP1* and *CYC1*. Graphs show individual values for each explant including the mean ± s.e.m. Note the discontinuous y axis. Explants A-E are taken within the para-macular region of the retina, M are samples dissected at the macular. Red data points highlight explants taken from 2 living donor eyes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression data with almost exclusive labelling within the RGC layer (RGCL) (Fig. 5A–C). Using single antibody staining, all DAPI$^+$ cells in the RGC layer were counted and the proportions of these cells that were positive for each RGC marker was quantified. In macular samples, 82.7 ± 1.9% of DAPI stained cells were NeuN$^+$, 80.9 ± 2.6% βIII Tubulin$^+$ and 81.8 ± 1.5% Thy-1$^+$ (Fig. 5Ai, Bi, Ci and Table 2). In para-macular regions, respective RGC markers were 53.7 ± 2.3%, 51.2 ± 0.6% and 52.8 ± 3.3% of all RGCL nuclei (Fig. 5Aii,

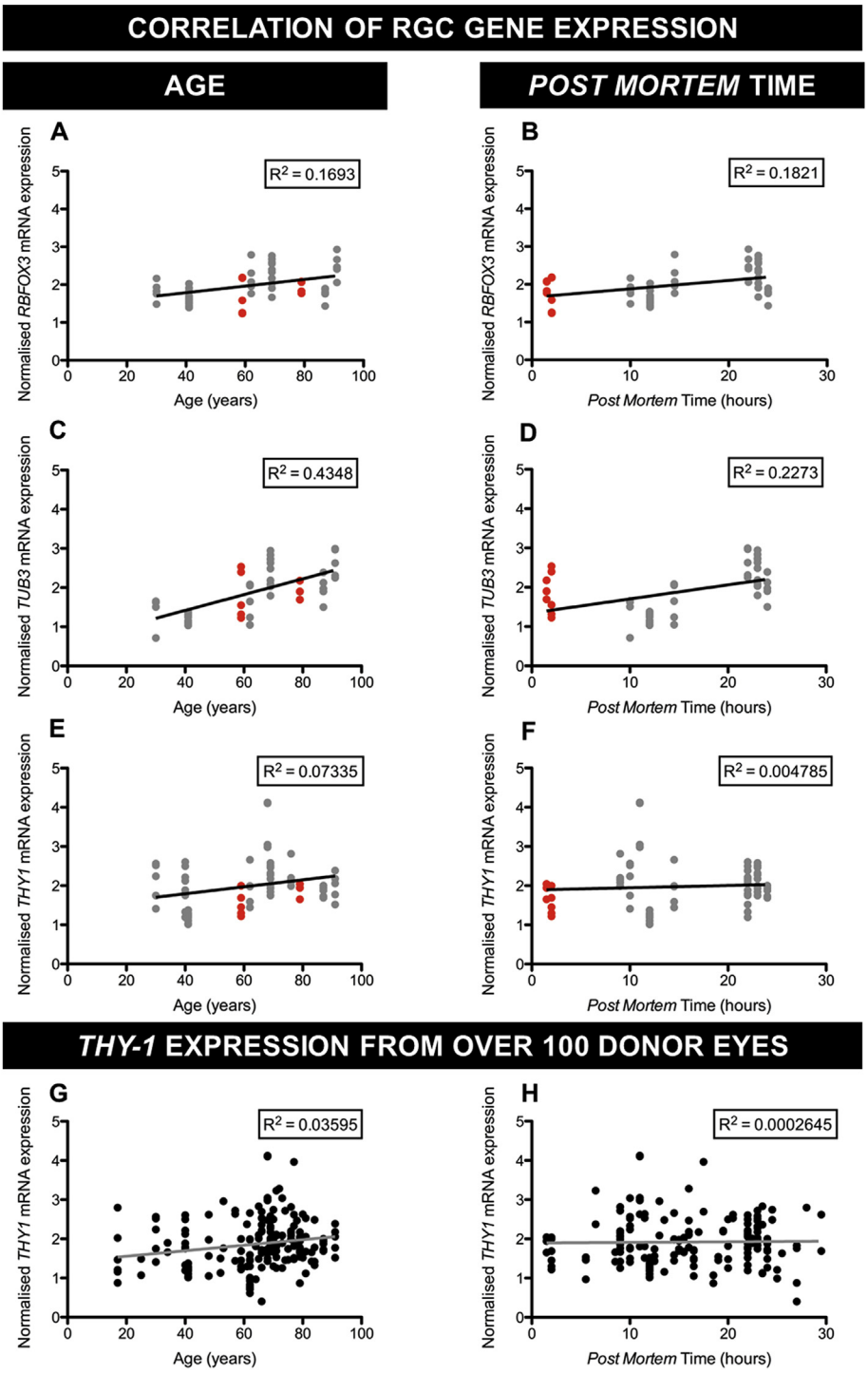


Fig. 3. RGC gene expression was not significantly influenced by the age of the donor or the time between donor death and retinal explant culture (*post mortem* time). (A–F) Each data point represents a single para-macular explant. There was no significant correlation between the expression of *RBFOX3* (n = 49 explants), *TUB3* (n = 49 explants) or *THY1* (n = 68 explants) with age or *post mortem* time. (G, H) *THY1* expression from a larger cohort of untreated eyes (n = 100 donor eyes) taken immediately after dissection similarly revealed no correlation between donor age, *post mortem* time and gene expression. All mRNA was normalised to the expression of the housekeeping genes *TOP1* and *CYC1*.

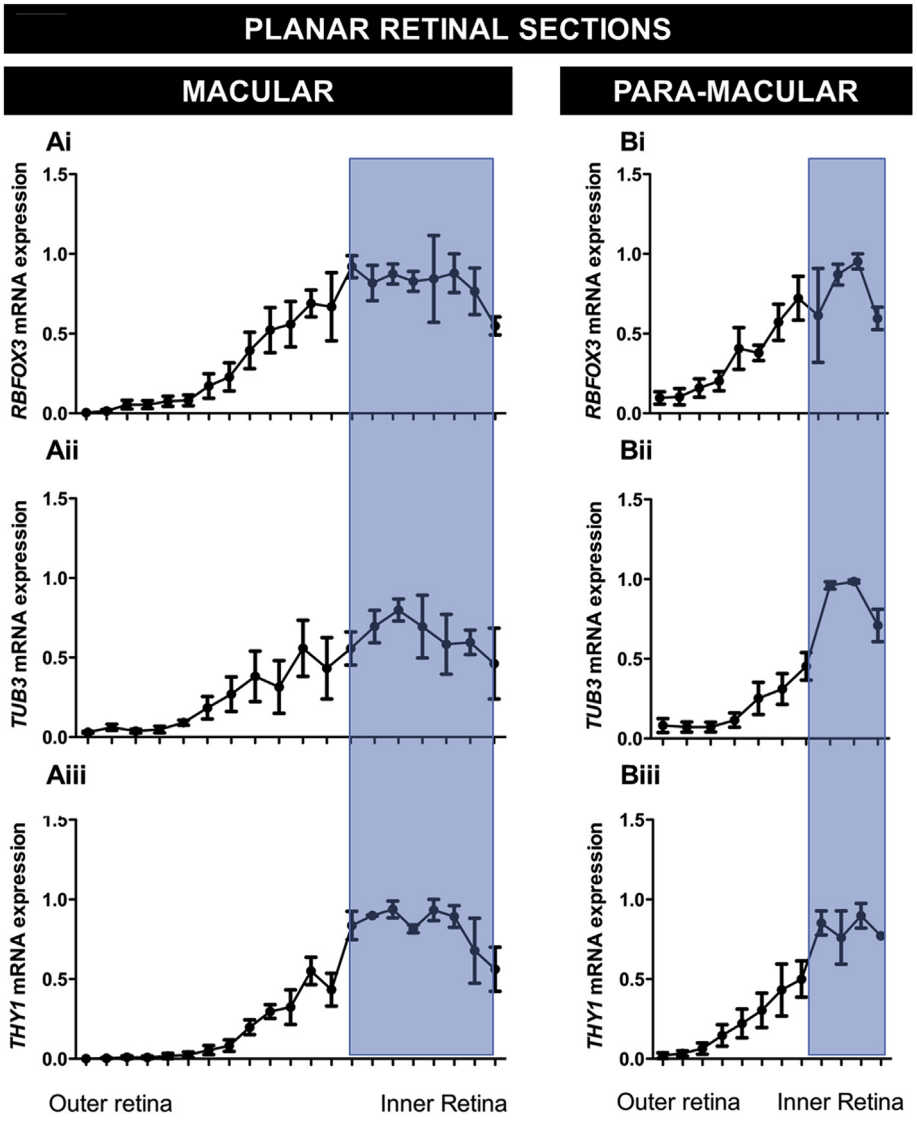


Fig. 4. Gene profiling revealed the location of RGC markers *RBFOX3*, *TUB3* and *THY1* throughout the retina in macular (A) and para-macular (B) explants. Up to 21 × 20 μm sections were collected throughout the entire retinal thickness in the plane of the retinal nuclear layers from the photoreceptor layer (left) to the retinal ganglion cell (RGC) layer (right). A greater number of sections were collected from macular explants due to the highest thickness in this region, with a broader RGC peak due to a thicker RGC layer. Similar expression patterns were seen between *RBFOX3*, *TUB3* and *THY1* in both macular and para-macular regions. (*RBFOX3* n = 3 donor eyes (for both macular and para-macular explants), *TUB3* n = 4 donor eyes, *THY1* n = 3 donor eyes; mean ± s.e.m).

Bii, Cii and Table 2). A small number of cells in the INL also stained positive for each antibody indicating likely displaced RGCs or amacrine cells (Fig. 5A–C).

Co-localisation between NeuN and βIII tubulin was then investigated to ensure that the RGC markers were binding to the same cell type within the RGCL. NeuN and βIII tubulin were selected over Thy-1 due to more complete and intense staining of the cell nuclei and soma, which improved evaluation. Both transverse section and whole-mount staining confirmed a strong level of co-localisation between the two markers with approximately 95% of NeuN$^+$ cells staining for βIII tubulin$^+$ (Fig. 6A–D and Table 3).

3.4. RGC survival with long-term culture

Having shown that NeuN could be used to accurately label RGCs in the human retina, the longevity of RGCs in para-macular retinal explants (HORCs) was assessed over a four-week period. NeuN immunohistochemistry was chosen as the preferred RGC marker to combine with apoptotic labelling due to its discrete staining of the cell body which enabled accurate quantification with the nuclear TUNEL staining. Changes in RGC gene expression were also assessed throughout the four-week period since these may be a more sensitive measure of RGC survival within explants (Niyadurupola et al., 2013). Of the three comparable RGC gene expression markers, we chose to investigate *THY1* to coincide with our previous research modelling short-term RGC degeneration (Niyadurupola et al., 2013, 2011; Osborne et al., 2015).

Over four weeks in culture, the overall appearance of HORCs showed no obvious structural changes, with explants retaining a considerable degree of histotypic organisation throughout long-term culture (Fig. 7A–E). Outer nuclear, inner nuclear and RGC nuclei were detectable in specific layers throughout the four-week time frame and NeuN$^+$ cells could be detected at all experimental time points.

Quantification of NeuN$^+$ cells at 1 week revealed no significant loss in SF and 10% FBS groups (Fig. 7F) although there was a

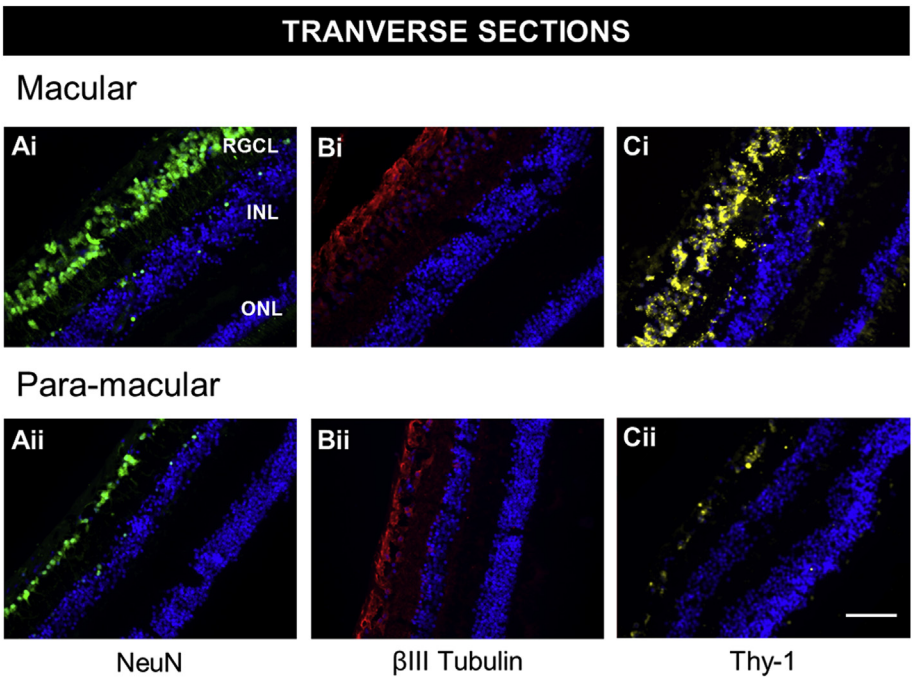


Fig. 5. Retinal ganglion cell (RGC) markers NeuN, βIII tubulin and Thy-1 labelled nuclei almost exclusively in the RGC layer of macular and para-macular explants (A–C). Para-macular explants contained a single row of RGCs that stained positive for each marker whilst macular samples had a denser RGC layer with multiple rows of NeuN, βIII tubulin and Thy-1 labelled cells. NeuN staining was located in the cell body (nucleus and surrounding cytoplasm), βIII tubulin labelled RGC soma and their processes and Thy-1 resulted in diffuse cell body staining. Scale = 50 μm (n = 4 donor eyes).

significant decrease in labelled cells at subsequent time points (23.0 ± 8.3% SF (n = 6 donor eyes), 20.8 ± 5.3% 10% FBS (n = 3 donor eyes) at week 4, $p \leq 0.05$, Fig. 7F). Greater RGC loss was measured in HORCs cultured in Neurobasal medium (NB) where there was a linear decrease in the number of $NeuN^+$ cells, decreasing approximately 25% (25.2 ± 4.2) at week 1 and by 60% (59.2 ± 7.5) at week 3 ($p \leq 0.05$, n = 3 donor eyes, Fig. 7F). Comparisons at 3 and 4 weeks revealed significantly fewer RGCs in explants cultured in NB medium compared to either SF or 10% FBS medium ($p \leq 0.05$, n = 3 donor eyes, Fig. 7F).

Assessing the viability of these cells using TUNEL labelling revealed that, although RGCs were detectable, the cells were undergoing time-dependent apoptosis. TUNEL-labelling showed a progressive increase in apoptotic labelling within the RGCL with time for all three treatment groups (Fig. 7G). Under all conditions, there was a significant increase in the number of $TUNEL^+$ NeuN–labelled nuclei at week 1 compared to week 0 ($n \geq 3$ donor eyes/treatment, $p \leq 0.05$, Fig. 7G). $TUNEL^+$ NeuN–labelling continued to increase at weekly intervals with almost all (82.2 ± 7.1 SF, 90.4 ± 0.8 10% FBS, 94.4 ± 2.8 NB) $NeuN^+$ cells staining as apoptotic by week 4 ($p \leq 0.05$, $n \geq 3$ donor eyes/treatment). HORCs taken from a living donor followed a similar trend (Fig. 7G). HORCs cultured in NB medium had the most rapid increase in TUNEL labelling (76.1 ± 11.2) with a significantly higher number of apoptotic RGCs compared to SF (45.0 ± 14.8) and 10% FBS (50.5 ± 12.1) groups at week 1 ($p \leq 0.05$, n = 3 donor eyes/treatment).

Table 2
The percentage of DAPI positive cells that stained positive for individual RGC markers in the RGCL (n = 4 donor eyes).

	NeuN	βIII tubulin	Thy-1
Macular	82.7 ± 1.9%	80.9 ± 2.6%	81.8 ± 1.5%
Para-macular	53.7 ± 2.3%	51.2 ± 0.6%	52.8 ± 3.3%

THY1 gene expression changes supported a decline in RGC viability with time in culture, shown by a rapid decrease in *THY1* immediately after dissection (week 0) to approximately 20% of the post-dissection level after 1 week (SF n = 5, 10% FBS n = 5, NB n = 2 donor eyes, Fig. 7H); expression levels remained at this 20% level for the duration of the experiments. An equivalent relative decline in *THY1* after 1 week was seen in both the retina of the living donor and *post mortem* material. Culturing explants in 10% FBS or NB did not prevent, or accelerate, *THY1* mRNA loss with time compared to SF culture (Fig. 7H).

4. Discussion

Organotypic culture models have become an increasingly popular experimental tool in retinal research, bridging the gap between *in vitro* and *in vivo* experimentation. We were the first to use the human retina to create a quantifiable explant model that can be used to study RGC degeneration (Niyadurupola et al., 2011). In the present study we further characterised markers for human RGCs and showed that *post mortem* human organotypic retinal cultures can be used as a long-term model to study RGC degeneration after axotomy.

4.1. RGC markers in the human retina

Firstly we revealed that neuronal markers for monitoring the RGC population in the rodent eye could be applied to the human retina, with precise and accurate labelling of cell populations within the RGCL. NeuN is widely regarded as being a useful RGC marker (Bull et al., 2011; Canola et al., 2007; Dijk et al., 2007; Zhong et al., 2007) and strong co-labelling has been detected between the antibody and fluorogold retrograde labelled mouse RGCs (Buckingham et al., 2008). Similarly, βIII tubulin has been shown to provide a reliable indicator of the number of surviving RGCs in

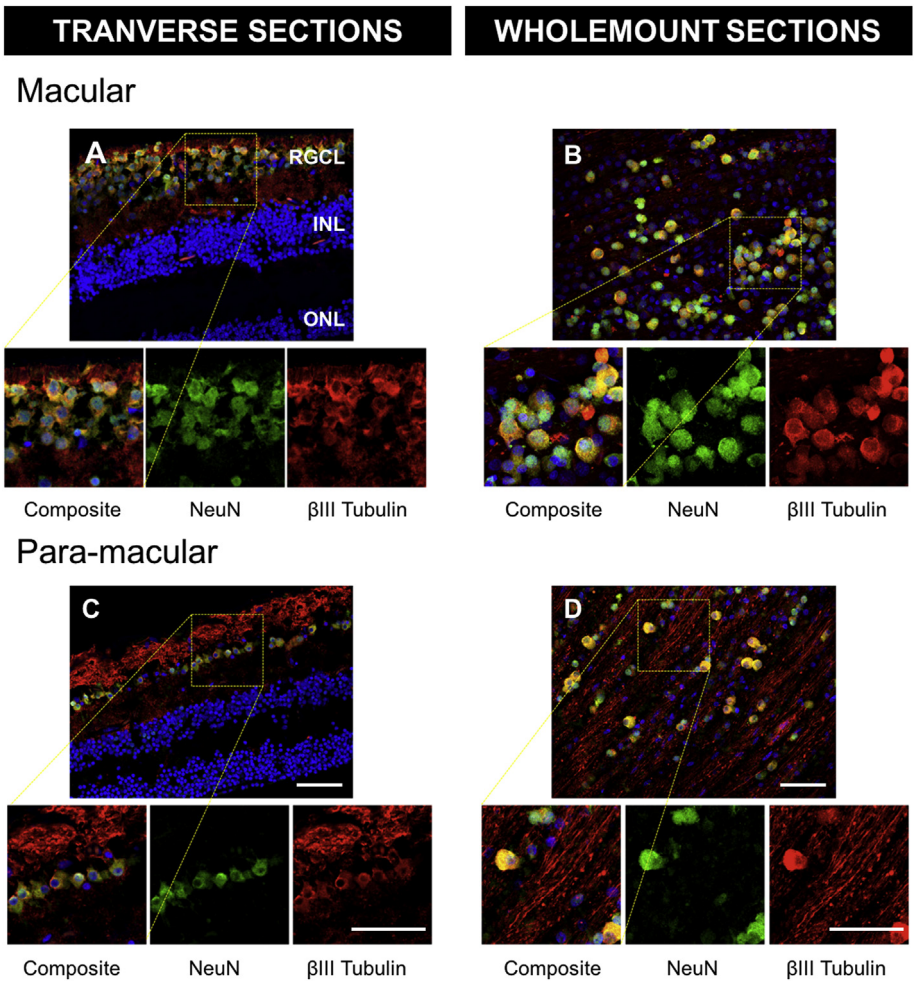


Fig. 6. Co-localisation of NeuN and βIII tubulin in transverse (A, C) and whole-mount (B, D) sections revealed both markers were specifically labelling the same cell type in the RGC layer. Similar levels of co-localisation could be seen in macular and para-macular regions of the retina. Scale = 50 μm (n = 5 donor eyes).

comparisons with fluorogold-labelled rat RGCs (Cui et al., 2003). Furthermore, Thy-1 transgenic animals have been used frequently in glaucoma research (Raymond et al., 2009) and *THY1* mRNA levels have been found by us (Niyadurupola et al., 2011) and others (Nash and Osborne, 1999) to provide a good index of RGC damage.

We initially showed that RGC mRNA expression across the macular and para-macular regions of the retina followed the typical RGC profile for the human retina, with greatest expression in the macula, the central region of the human retina which is responsible for the highest visual acuity. The mRNA expression results were in keeping with our previous data, which showed that *RBFOX3 (NeuN)* mRNA decreased from the central macula to the periphery (Niyadurupola et al., 2011). We further demonstrated that, for each of the three RGC markers investigated, the same expression profile was identified and exhibited a consistent organisation throughout the retina, with minimal expression in the outer retina increasing towards maximal expression in the innermost retina where the RGC cell bodies are located.

The mRNA expression data was supported by immunohistochemistry for NeuN, βIII tubulin and Thy-1 that only labelled cells in the inner retina. NeuN staining was located in the cell body (nucleus and surrounding cytoplasm) as has been shown previously (Bull et al., 2011; Raymond et al., 2008) whilst βIII tubulin labelled RGC soma and their processes, similar to the distribution observed in rodent retinas (Johnson and Martin, 2008; Li et al., 2010). Thy-1 is a cell surface glycoprotein that associates with the RGC bodies, dendrites and axons (Nash and Osborne, 1999; Osborne and Larson, 1996; Sheppard et al., 1991) and in our studies labelling was observed primarily on the cell bodies. However labelling was diffuse, probably because Thy-1 is a cell surface marker and resulted, therefore, in Thy-1 being the most challenging to quantify.

Co-localisation of NeuN and βIII tubulin confirmed that either antibody could be used as a reliable marker to quantify RGCs. Others have shown that a co-localisation pattern also exists for Thy-1 and NeuN whereby 95.4% of thy-1-CFP cells in the RGCL also labelled with NeuN (Raymond et al., 2008). In addition, we observed a greater proportion of NeuN, βIII tubulin and Thy-1 positive cells within the RGCL of macular samples compared to para-macular samples; this would be in keeping with the functional superiority of the central region of the retina with previous estimates suggesting that almost all cells in the RGCL at the human

Table 3
The percentage of NeuN labelled cells that were also βIII tubulin positive in macular and para-macular explants (n = 5 donor eyes).

	Co-localisation between NeuN and βIII tubulin
Macular	95.0 ± 2.3%
Para-macular	93.4 ± 3.0%

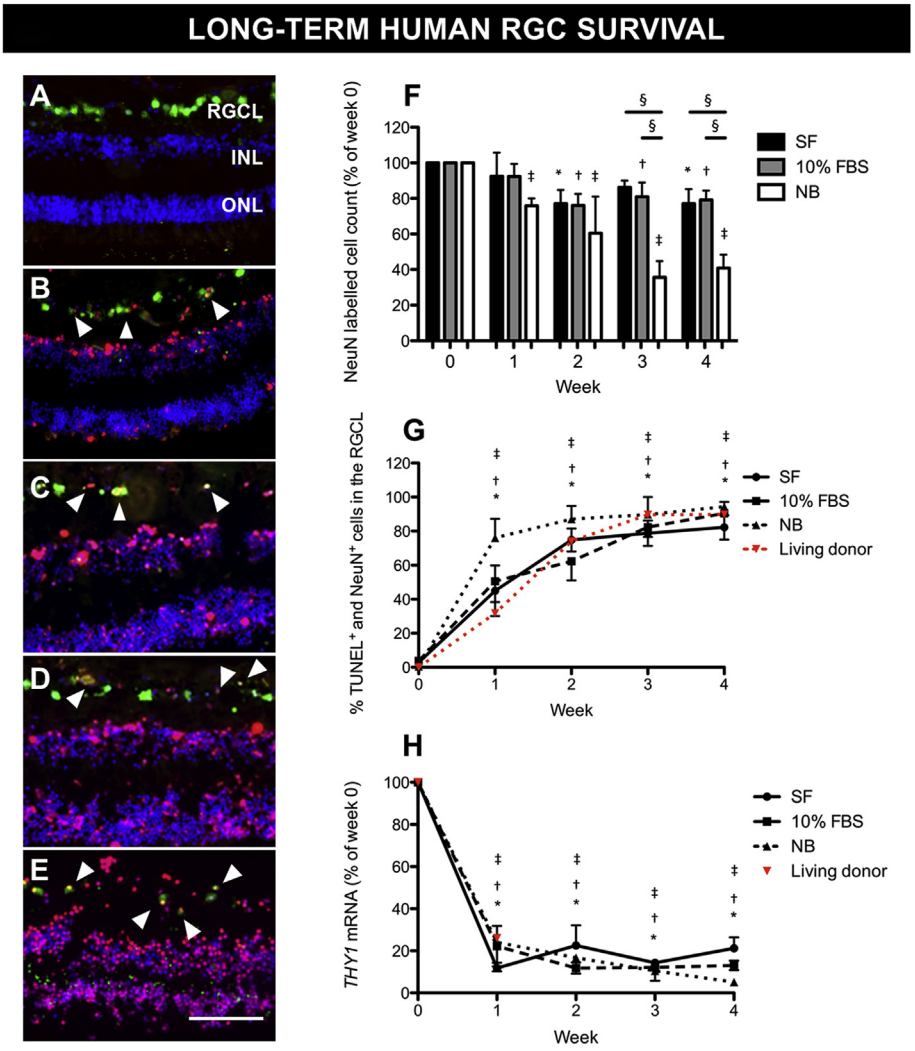


Fig. 7. The survival of human retinal ganglion cells (RGCs) in *ex vivo* para-macular explants (HORCs) over 4 weeks. Representative immunofluorescence photomicrographs of HORCs cultured in SF medium at (A) 0 weeks post dissection, (B) 1 week, (C) 2 week, (D) 3 week, (E) 4 week. (F) The percentage of $NeuN^+$ cells in the RGC layer (mean ± s.e.m; n = 3–6 donor eyes) relative to same donor counts immediately post dissection. (G) The proportion of $TUNEL^+$ $NeuN^+$ cells in the RGC layer (mean ± s.e.m; n = 3–6 donor eyes) with time in culture. (H) The change in *THY1* mRNA expression (mean ± s.e.m; n = 2–6 donor eyes) with time in culture relative to same donor expression immediately after explant creation. DAPI = blue, NeuN = green, TUNEL = red. White arrows highlight $TUNEL^+$ $NeuN^+$ cells in the RGC layer. GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer. Red data points = Living donor explants cultured in SF medium. Scale = 50 μm *, †, ‡ indicates a statistically significant differences compared to samples processed immediately after dissection in SF, 10% FBS and Neurobasal (NB) medium respectively. § indicates a significant difference between SF and NB results at the same time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fovea are RGCs (only 3% representing displaced amacrine cells). In para-macular samples the proportion of RGCs in the RGCL decreased to approximately 60%, a similar percentage to that seen in mouse and rat retina; whereby approximately 50–60% of cells were RGCs with the remainder being of amacrine origin (Jeon et al., 1998; Perry, 1981). Although we did not measure changes at the periphery, others have shown that the proportion of RGCs can decrease to as few as 20%, with the remainder being of amacrine lineage (Curcio and Allen, 1990). Sampling at the periphery therefore may contain too few RGCs for quantitative neurodegeneration studies.

It should be noted that in rodents βIII tubulin antibodies can cross react with ligands expressed on amacrine cells (Sharma and Netland, 2007). Similarly, probing for NeuN in rats revealed significant INL staining, likely to be that of amacrine cells (Bull et al., 2011; Johnson et al., 2014). However, these latter observations were less apparent in the human retina with very few NeuN or βIII tubulin-labelled cells being identified outside the RGCL. Our results also showed that the proportions of $NeuN^+$ and βIII $tubulin^+$ cells closely matched those that were Thy-1^+ which has been reported by others to exclusively label RGCL cells (Kwong et al., 2010; Liu et al., 2013) or with expression levels several fold higher on RGCs than amacrine cells (Raymond et al., 2009). Furthermore, we believe the appropriateness of these markers can be shown by the differential expression between macular and para-macular regions; it is likely that regional differences would not be seen if labelling was inclusive of amacrine cells. The retinal profiles of *THY1* and the cholinergic amacrine marker *ChAT* also differed in peak expression indicating a likely difference in cell type detection and that we were accurately measuring just RGC cells (Supplementary Fig. 1).

4.2. The suitability of using post mortem human retinal tissue

There has been an assumption that it is not feasible to utilise *post mortem* retina as a model of degeneration due to the extended time from death to retinal isolation. Furthermore, whole-mount

retinal analysis has demonstrated previously that RGC number in the human retina can vary considerably between individuals, particular with age (Harman et al., 2000). However, the present results have indicated that RGC mRNA expression (the most sensitive indicator of RGC loss) in the area surrounding the macula does not show a large degree of variability with age or *post mortem* time. Even when eyes were taken with a very short transit time (<2 h) from living donors, we did not see a substantial difference in RGC mRNA expression compared to aged, long (up to 24 h) *post-mortem* time eyes. The rates of *THY1* mRNA loss and apoptotic labelling also revealed little difference between donors. Thus it can be concluded that a rapid degradation of RGC marker mRNA did not occur between death/removal of eyes and dissection. Additionally the degeneration of RGCs from living donors occurred at the same speed.

4.3. Human retinal explants as a model of chronic neurodegeneration

Our final experiments assessed the feasibility of using adult human retina as a long-term culture system for studying RGC degeneration. Of the three RGC markers investigated, NeuN proved quickest, easiest and most reliable to count. Using antibodies to βIII tubulin and Thy-1 led to additional labelling of RGC dendrites and axons making quantification more time consuming. βIII tubulin and Thy-1 have also proven difficult to assess in rat explants (Bull et al., 2011) and therefore might not be appropriate for large scale analyses. Interestingly, it has been shown that Thy-1 (Huang et al., 2006) and βIII tubulin expression are down regulated following injury, prior to cell loss, which could make NeuN an appropriate RGC marker for immunohistochemical identification and quantification, although the others may be useful therefore in assessing earlier neurodegenerative changes.

As a model of degeneration, retinal explants require complete RGC axotomy, an insult that ultimately leads to RGC death in *in vivo* models (Levkovitch-Verbin et al., 2013; Wang et al., 2015). The progressive nature of RGC loss, therefore, can provide a useful model in which to investigate the neurodegenerative features of optic nerve degeneration associated with injury or glaucoma.

In our experiments, human explants showed progressive RGC apoptotic labelling with time, with minimal damage immediately post dissection, as observed previously (Niyadurupola et al., 2011), to extensive RGC apoptotic labelling at 4 weeks. The rate of apoptosis closely matched that seen in animal optic nerve crush models (70% of RGCs are apoptotic within the first 14 days after injury (Maghariou et al., 2011)), although HORC RGC death appeared to be faster than in rodent explant models (Johnson and Martin, 2008; McKernan et al., 2007). It is unlikely that the species differential effect is due to the extended *post mortem* times seen when using human organotypic cultures because: 1) apoptosis at the beginning of experiments was absent and 2) freshly prepared living donor explants followed a similar rate of cell death to *post mortem* explants. One possibility is that the mediums used did not contain the necessary growth factors to maintain human RGC survival for such extended times and this warrants further investigation. Support for the assumption that medium composition is important for survival was shown in the experiments where explants displayed variations in the rate of neurodegeneration depending on medium composition. After we had identified that serum did not improve survival in human explants, we decided to test an alternative SF medium in the hope that this might enhance survival. We opted for the most commonly used medium composition for the survival of RGCs (Bull et al., 2011; Johnson and Martin, 2008) which is also a medium shown to enhance hippocampal neuron survival over 4 weeks in culture (Brewer, 1997); this was Neurobasal®-A with the stated supplements. However to our surprise, we observed a rapid loss of RGCs and increased early apoptosis in the HORCs cultured in Neurobasal medium compared to those in SF DMEM/F12 medium. The reason for the survival differences with the two media was unclear and beyond the scope of this paper, however we can speculate that differences in amino acids, vitamins, ferric nitrate and sodium pyruvate compositions might have played a role, particularly a lack of glutamate and aspartate in NB medium. Additionally, the B27 supplement in NB medium can suppress the growth of glial cells (Brewer et al., 1993), which may dampen the supportive role of these cells in human explants. Identification as to why NB was detrimental to human RGCs would be useful for future investigation although the result does itself highlight that cell soma delivered trophic factors are important in affecting RGC degeneration.

4.4. Further uses of human explants

Although TUNEL labelling was only quantified in the RGC layer, an overall increase in TUNEL labelling could be seen throughout the retina. With apoptosis evident in the INL and ONL, and appearing to increase with time in culture, use of human organotypic cultures may prove useful in the study of other retinal diseases. Rat organotypic cultures, for example, are currently being used to measure growth factor neuroprotection of photoreceptors (Lipinski et al., 2011). Assessing novel neuroprotective strategies relating to retinal neurodegeneration of either photoreceptors (e.g. in age-related macula degeneration or retinitis pigmentosa) or RGCs (e.g. in glaucoma) could be a useful application for this model.

5. Conclusions

Strong similarity between human *post mortem* tissue and living donor retina suggest that human retinal explants could be a valuable resource as a chronic experimental model of RGC degeneration. Various markers can be used to accurately quantify RGC number throughout the retina and alterations to the medium can have an impact on the rate of apoptosis and cell loss. The human explant model therefore provides a platform to investigate future neuroprotective and regenerative therapies, which rely on delaying RGC apoptosis.

Competing interests

No competing interests declared.

Author contribution

JS and A.O. conceived this study. A.O. and J.S. designed the experiments. A.O., M.H. and P.W. performed the experiments. A.O., M.H. and P.W. analysed the data. A.O. and J.S. interpreted the data being published. The manuscript was written by A.O. and edited by J.S. and D.C.B.

Funding

The Edith Murphy Foundation, The International Glaucoma Association, The Norwich Glaucoma Research Fund, AstraZeneca, University of East Anglia, Faculty of Science and The Humane Research Trust.

Acknowledgements

The authors would like to express their gratitude to all eye donors, Pamela Keeley, Mary Tottman and Samantha Major (of the

East Anglian Eye Bank) for donor eye retrieval and both Bridget Hemmant and Marc Moncrief (of the Norfolk and Norwich University Hospital) for providing living donor samples.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2015.09.012.

References

Albrecht May, C., 2008. Comparative anatomy of the optic nerve head and inner retina in non-primate animal models used for glaucoma research. Open Ophthalmol. J. 2, 94–101.

Bobu, C., Lahmam, M., Vuillez, P., Ouarour, A., Hicks, D., 2008. Photoreceptor organisation and phenotypic characterization in retinas of two diurnal rodent species: potential use as experimental animal models for human vision research. Vis. Res. 48, 424–432.

Brewer, G., 1997. Isolation and culture of adult rat hippocampal neurons. J. Neurosci. Methods 71, 143–155.

Brewer, G.J., Torricelli, J.R., Evege, E.K., Price, P.J., 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res. 35, 567–576.

Buckingham, B., Inman, D., Lambert, W., Oglesby, E., Calkins, D., Steele, M., Vetter, M., Marsh-Armstrong, N., Horner, P., 2008. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. J. Neurosci. 28, 2735–2744.

Bull, N., Johnson, T., Welsapar, G., DeKorver, N., Tomarev, S., Martin, K., 2011. Use of an adult rat retinal explant model for screening of potential retinal ganglion cell neuroprotective therapies. Invest. Ophthalmol. Vis. Sci. 52, 3309–3320.

Buyens, T., Gaublomme, D., Van Hove, I., De Groef, L., Moons, L., 2014. Quantitative assessment of neurite outgrowth in mouse retinal explants. Methods Mol. Biol. 1162, 57–71.

Caffé, A., Ahuja, P., Holmqvist, B., Azadi, S., Forsell, J., Holmqvist, I., Söderpalm, A., van Veen, T., 2001. Mouse retina explants after long-term culture in serum free medium. J. Chem. Neuroanat. 22, 263–273.

Canola, K., Angénieux, B., Tekaya, M., Quiambao, A., Naash, M., Munier, F., Schorderet, D., Arsenijevic, Y., 2007. Retinal stem cells transplanted into models of late stages of retinitis pigmentosa preferentially adopt a glial or a retinal ganglion cell fate. Invest. Ophthalmol. Vis. Sci. 48, 446–454.

Cui, Q., Yip, H., Zhao, R., So, K., Harvey, A., 2003. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. Mol. Cell. Neurosci. 22, 49–61.

Curcio, C., Allen, K., 1990. Topography of ganglion cells in human retina. J. Comp. Neurol. 300, 5–25.

Dijk, F., Bergen, A., Kamphuis, W., 2007. GAP-43 expression is upregulated in retinal ganglion cells after ischemia/reperfusion-induced damage. Exp. Eye Res. 84, 858–867.

Donovan, S., Dyer, M., 2006. Preparation and square wave electroporation of retinal explant cultures. Nat. Protoc. 1, 2710–2718.

Engelsberg, K., Ehinger, B., Ghosh, F., 2008. Early development of retinal subtypes in long-term cultures of human embryonic retina. Curr. Eye Res. 33, 185–191.

Fernandez-Bueno, I., Fernández-Sánchez, L., Gayoso, M., García-Gutierrez, M., Pastor, J., Cuenca, N., 2012. Time course modifications in organotypic culture of human neuroretina. Exp. Eye Res. 104, 26–38.

Harman, A., Abrahams, B., Moore, S., Hoskins, R., 2000. Neuronal density in the human retinal ganglion cell layer from 16–77 years. Anat. Rec. 260, 124–131.

Huang, W., Fileta, J., Guo, Y., Grosskreutz, C., 2006. Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. Curr. Eye Res. 31, 265–271.

Jeon, C., Strettoi, E., Masland, R., 1998. The major cell populations of the mouse retina. J. Neurosci. 18, 8936–8946.

Johnson, E., Doser, T., Cepurna, W., Dyck, J., Jia, L., Guo, Y., Lambert, W., Morrison, J., 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, 504–518.

Johnson, T., Dekorver, N., Levasseur, V., Osborne, A., Tassoni, A., Lorber, B., Heller, J., Villasmil, R., Bull, N., Martin, K., et al., 2014. Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain 137, 503–519.

Johnson, T., Martin, K., 2008. Development and characterization of an adult retinal explant organotypic tissue culture system as an in vitro intraocular stem cell transplantation model. Invest. Ophthalmol. Vis. Sci. 49, 3503–3512.

Kwong, J., 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.

Levkovitch-Verbin, H., 2004. Animal models of optic nerve diseases. Eye Lond. 18, 1066–1074.

Levkovitch-Verbin, H., Makarovsky, D., Vander, S., 2013. Comparison between axonal and retinal ganglion cell gene expression in various optic nerve injuries including glaucoma. Mol. Vis. 19, 2526–2541.

Li, Y., Zheng, H., Liu, P., Chen, Y., She, Z., Xiao, H., Zhou, G., 2010. The new targets of ouabain in retinal interneurons of Sprague-Dawley rats. Brain Res. Bull. 81, 617–624.

Lipinski, D., Singh, M., MacLaren, R., 2011. Assessment of cone survival in response to CNTF, GDNF, and VEGF165b in a novel ex vivo model of end-stage retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. 52, 7340–7346.

Liu, B., Sun, X., Suyeoka, G., Garcia, J., Leiderman, Y., 2013. TGFβ signaling induces expression of Gadd45b in retinal ganglion cells. Invest. Ophthalmol. Vis. Sci. 54, 1061–1069.

Magharious, M., D'Onofrio, P., Hollander, A., Zhu, P., Chen, J., Koeberle, P., 2011. Quantitative iTRAQ analysis of retinal ganglion cell degeneration after optic nerve crush. J. Proteome Res. 10, 3344–3362.

McKernan, D.P., Guerin, M.B., O'Brien, C.J., Cotter, T.G., 2007. A key role for calpains in retinal ganglion cell death. Invest. Ophthalmol. Vis. Sci. 48, 5420–5430.

Nash, M.S., Osborne, N.N., 1999. Assessment of Thy-1 mRNA levels as an index of retinal ganglion cell damage. Invest. Ophthalmol. Vis. Sci. 40, 1293–1298.

Niyadurupola, N., Sidaway, P., Ma, N., Rhodes, J.D., Broadway, D.C., Sanderson, J., 2013. P2X7 receptor activation mediates retinal ganglion cell death in a human retina model of ischemic neurodegeneration. Invest. Ophthalmol. Vis. Sci. 54, 2163–2170.

Niyadurupola, N., Sidaway, P., Osborne, A., Broadway, D.C., Sanderson, J., 2011. The development of human organotypic retinal cultures (HORCs) to study retinal neurodegeneration. Br. J. Ophthalmol. 95, 720–726.

Osborne, A., Aldarwesh, A., Rhodes, J., Broadway, D.C., Everitt, C., Sanderson, J., 2015. Hydrostatic pressure does not cause detectable changes in survival of human retinal ganglion cells. PLoS One. 10, e0115591.

Osborne, N.N., Larson, A., 1996. Antigens associated with specific retinal cells are affected by ischaemia caused by raised intraocular pressure: effect of glutamate antagonists. Neurochem. Int. 29, 263–270.

Perry, V., 1981. Evidence for an amacrine cell system in the ganglion cell layer of the rat retina. Neuroscience 6, 931–944.

Raymond, I., Pool, A., Vila, A., Brecha, N., 2009. A Thy1-CFP DBA/2J mouse line with cyan fluorescent protein expression in retinal ganglion cells. Vis. Neurosci. 26, 453–465.

Raymond, I., Vila, A., Huynh, U., Brecha, N., 2008. Cyan fluorescent protein expression in ganglion and amacrine cells in a thy1-CFP transgenic mouse retina. Mol. Vis. 14, 1559–1574.

Rojas, C.V., Martínez, J.I., Flores, I., Hoffman, D.R., Uauy, R., 2003. Gene expression analysis in human fetal retinal explants treated with docosahexaenoic acid. Invest. Ophthalmol. Vis. Sci. 44, 3170–3177.

Sharma, R., Netland, P., 2007. Early born lineage of retinal neurons express class III beta-tubulin isotype. Brain Res. 1176, 11–17.

Sheppard, A.M., Konopka, M., Robinson, S.R., Morgan, I.G., Jeffrey, P.L., 1991. Thy-1 antigen is specific to ganglion cells in chicks. Neurosci. Lett. 123, 87–90.

Wang, X., Lin, J., Arzeno, A., Choi, J., Boccio, J., Frieden, E., Bhargava, A., Maynard, G., Tsai, J., Strittmatter, S., 2015. Intravitreal delivery of human NgR-Fc decoy protein regenerates axons after optic nerve crush and protects ganglion cells in glaucoma models. Invest. Ophthalmol. Vis. Sci. 56, 1357–1366.

Zhang, X., Li Liu, D., Chiang, S., Choy, K., Pang, C., Lam, D., Yam, G., 2010. Immunopanning purification and long-term culture of human retinal ganglion cells. Mol. Vis. 16, 2867–2872.

Zhong, L., Bradley, J., Schubert, W., Ahmed, E., Adamis, A., Shima, D., Robinson, G., Ng, Y., 2007. Erythropoietin promotes survival of retinal ganglion cells in DBA/2J glaucoma mice. Invest. Ophthalmol. Vis. Sci. 48, 1212–1218.