THE EFFECT OF DIFFERENT MICROGLIAL ACTIVATION STATES ON THE SURVIVAL OF RETINAL GANGLION CELLS

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EFFECT OF DIFFERENT MICROGLIAL ACTIVATION STATES ON RGC SURVIVAL

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ABSTRACT

Purpose: Microglia are the innate immune cells of the central nervous system. Activated microglia release nitric oxide, glutamate, and superoxide radicals, which are harmful to retinal ganglion cells (RGCs). They may also benefit surviving cells by removing toxic cellular debris or by secretion of neurotrophic factors. The paradoxical role of microglia remains controversial because the nature and time-course of the injury that determines whether microglia acquire a neuroprotective or pro-inflammatory phenotype is unknown. HAPI cells are an immortalized microglial cell line, whose phenotype can be manipulated *in vitro*. It is my HYPOTHESIS that pharmacological manipulation of microglia to acquire either a pro-inflammatory or pro-survival phenotype will exacerbate neuronal cell death or enhance neuronal survival after injury, respectively.

Method: Lipopolysaccharides (LPS) were used to hyper-stimulate the HAPI cells and minocycline to maintain the HAPI cells in a quiescent state. Prior to the experiments, the HAPI cells were labelled with Wheat Germ Agglutinin conjugated to Texas Red. The HAPI cells were cultured and exposed to minocycline (10 µg/mL for 1 hour) or LPS (1 µg/mL for 24 hours). Sprague-Dawley rats then recieved intraocular (30,000 cells) or tail vein (5 million cells) injections of either the minocycline treated HAPI cells or the LPS treated HAPI cells and an optic nerve crush. Retinas were examined at 4-14 days later and the number of surviving RGCs will be determined by Brn3a labelling of RGCs. BM88 antibody labelling was done to determine the severity of the injury and to determine molecular changes after neuroinflammation.

Results: Injection of untreated HAPI cells resulted in the greater loss of RGCs early after ONC when injected into the vitreous and later after ONC when injected into the tail vein. LPS activated HAPI cells injected into the vitreous resulted in greater RGC loss with and without injury. When injected into the tail vein with ONC there was no loss of RGCs 4 days after ONC but later there was greater loss of RGCs. Minocycline treated HAPI cells injected into the vitreous resulted in greater RGC survival than when untreated HAPI cells were injected. However, when injected into the tail vein with ONC there was greater loss of RGCs. There was also BM88 down regulation after injury and this was more pronounced after HAPI cell injection.

Conclusion: Neuroprotection or cytotoxicity of microglia depends on the type of activation, time course of the injury, and if the microglia act on the axon or cell body of the retinal ganglion cell.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine-5'-triphosphate
BAD	Bcl2 associated death promoter
BAX	Bcl-2–associated X protein
BBB	Blood brain barrier
BDNF	Brain dervied neurotrophic factor
bFGF	Basic fibroblast growth factor
BMDM	Bone marrow derived microglia
CAG	Closed angle glaucoma
Cend1	Cell cycle exit and neuronal differentiation
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSPG	Chondroitin sulphate proteoglycans
Cx3cl 1	Chemokine fractalkine
ECM	Extracellular matrix
ERK	Extracellular-signal-regulated kinases
EU	Endotoxin unit
FG	Fluorogold
GCL	Ganglion cell layer
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial-fibrillary acidic protein
GFP	Green fluorescent protein
HAPI	Highly aggressive proliferating immortalized microglial cell line
HSP	Heat shock protein
HTG	High tension glaucoma
Iba-1	Ionized calcium binding adapter molecule 1
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
INL	Inner nuclear layer
IOP	Intraocular pressure
IP3	Inositol 1,4,5-triphosposte
IPL	Inner plexiform layer
IV	Intravitreal
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharides

M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
Mino	Minocycline
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
mSOD	Mutant superoxide dismutase
MTPT	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drugs
NTG	Normal tension glaucoma
OAG	Open angle glaucoma
OG	Oregon green
ONC	Optic nerve crush
ONL	Outer nuclear layer
ONT	Optic nerve transection
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PRR	Pattern recognition receptors
RGC	Retinal ganglion cell
rMC-1	Immortalized retinal Müller cell line 1
ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
SVZ	Subventricular zone
TDGF	Target derived growth factors
TGF β	Transforming growth factor β
TLR	Toll like receptor
TNF	Tumor necrosis factor
TR	Texas red
TrkB	Tyrosine kinase receptor B
TV	Tail vein
UPS	Ubiquitin proteasome system
UTP	Uridine-5'-triphosphate
WGA	Wheat germ aggulutinin

CHAPTER 1: GENERAL INTRODUCTION

1.1 Neuroinflammation in CNS injury and Disorders

Neuroinflammation has recently become a hot topic in neuroscience, having its own dedicated journals and subsections at neuroscience meetings. For many years, the central nervous system (CNS) was thought to be an immune privileged environment that was not accessible to the systemic immune system or susceptible to inflammation (Lucas, Rothwell, & Gibson, 2006). Inflammation in the CNS is easy to overlook because it is atypical and occurs either with delayed or without leucocyte infiltration (Lucas et al., 2006; Minghetti, 2005; Perry, Cunningham, & Boche, 2002). It is now widely accepted that although the CNS differs in way it reacts to injury and pathogens compared to other tissues, there are key inflammatory responses that occur (see table 1.1) (Lucas et al., 2006). Neuroinflammation has recently been implicated as an important process in neurodegenerative disorders, such as Alzheimer ’s disease, Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and glaucoma (Consilvio, Vincent, & Feldman, 2004; Dalrymple et al., 2007; Minghetti, 2005; O'Callaghan, Sriram, & Miller, 2008; Qian, Flood, & Hong, 2010; Tezel et al., 2007). However, it is argued if neuroinflammation is the cause or result of the neurodegenerative disease (Bauer, Kerr, & Patterson, 2007; O'Callaghan et al., 2008; Thameem Dheen, Kaur, & Ling, 2007).

1.2 Definition of Neuroinflammation

Neuroinflammation is a term that is not easy to define because inflammation in the CNS is atypical. Normal inflammation in non-CNS tissue is

characterized by four cardinal signs: *rubor et tumor cum calore et dolore* (swelling, redness, heat, and pain) (Medzhitov, 2010). The mechanism of inflammation has now been described in more detail to include invasion of circulating immune cells (such as monocytes, lymphocytes and macrophages) and the induction of cytokines, kinins, and cyclooxygenase products (Lucas et al., 2006; O'Callaghan et al., 2008). There are four components to the inflammatory response that include inflammatory inducers, sensors to detect the inducers, the inflammatory mediators produced by the sensors, and the target tissue that is affected (figure 1.1) (Medzhitov, 2010). Depending on the nature of the trigger, there is a distinct response. When a bacterial pathogen is recognized by receptors of the innate immune system (i.e. Toll-like receptors on macrophages; TLRs), there is a production of pro-inflammatory cytokines (such as tumor necrosis factor and IL-1), chemokines (such as CCL2), and prostaglandins that targets the affected tissue (Medzhitov, 2010). This results in vasodilation, extravasation of neutrophils, and leakage of plasma into the infected tissue (Medzhitov, 2010). Recruitment of neutrophils, macrophages, and mast cells results in the destruction of the pathogen. There is also production of acute phase proteins by liver cells that triggers brain endothelium to produce prostaglandins and leads to sickness behaviour (fever, fatigue, and social withdrawal) (Medzhitov, 2010; Pecchi, Dallaporta, Jean, Thirion, & Troadec, 2009). Depending on the type of infection, the sensor, mediators, and target tissue can change, such as with viral infection that is characterized by interferon and activation of lymphocytes, whereas

parasitic worms leads to histamine production and activation of basophils (Medzhitov, 2010).

When there is just tissue damage without infection, inflammation aims to promote tissue repair and prevent infection by breaking down the extracellular matrix (ECM), generating reactive oxygen species (ROS), activating tissue-resident macrophages, and recruiting neutrophils and monocytes (Medzhitov, 2010; O'Callaghan et al., 2008). This concept of inflammation is typically extended to the CNS and results in gliosis, which is the activation of microglia and astrocytes (Kreutzberg, 1996; O'Callaghan & Sriram, 2005; O'Callaghan et al., 2008). Gliosis is still not well understood but there are many pro-inflammatory cytokines and chemokines produced in the process (O'Callaghan et al., 2008; Pekny & Nilsson, 2005).

1.2.1 Gliosis

After injury to the CNS, reactive gliosis occurs in order to seal off the injured tissue and restrict inflammation and neuronal death (Buffo et al., 2008; Sofroniew, 2005). Cells that participate in reactive gliosis include astrocytes, microglia, oligodendrocyte precursor cells, meningeal cells, and stem cells (Buffo et al., 2008; Fawcett & Asher, 1999). These cells arrive at different times after CNS injury. Within a few hours of the injury, resident microglia migrate to the injury site (Buffo et al., 2008). Microglia are the innate immune cells of the CNS and are a key facilitator of neuroinflammation (Streit, Mrak, & Griffin, 2004). Approximately 3 days after the injury, monocytes and macrophages from

the blood are recruited to the affected area (Koshinaga & Whittemore, 1995). In contrast, this happens after a few hours of injury in the peripheral nervous system (PNS), which may partially explain why the PNS regenerates more than the CNS (Koshinaga & Whittemore, 1995). Oligodendrocyte precursors and meningeal cells (if the injury effects the meninges) will migrate to the injury site between 3-5 days after CNS injury (Buffo et al., 2008). Oligodendrocyte precursors may be involved in repairing and remyelination after injury (Chang, Nishiyama, Peterson, Prineas, & Trapp, 2000; M. T. Fitch & Silver, 2008; Nishiyama, 2007; Wilson, Scolding, & Raine, 2006). Astrocytes become activated and migrate to the injury slowly and are the final component of the glial scar (Buffo et al., 2008; Okada et al., 2006). There are also molecular and morphological changes that occur in astrocytes after injury, such as hypertrophy of their somas and processes, increased expression of glial-fibrillary acidic protein (GFAP), and re-expression of progenitor markers vimentin and nestin (Alonso, 2005; Buffo et al., 2008; Miyake, Hattori, Fukuda, Kitamura, & Fujita, 1988; Sofroniew, 2005; Wilhelmsson et al., 2006). Astrocytes are important for maintaining the blood brain barrier (BBB), neurotransmitter and ion regulation, and the production of the extracellular matrix (ECM) (Busch & Silver, 2007; Massey et al., 2006; Risau & Wolburg, 1990; Schousboe & Westergaard, 1995; Tom, Doller, Malouf, & Silver, 2004; Walz, 1989). Müller cells are radial astrocytes in the retina that participate in a number of physiological events related to signal transmission by neurons and also involved in gliosis (Dyer & Cepko, 2000; Humphrey, Constable,

Chu, & Wiffen, 1993; MacLaren, 1996). In response to injury, Müller cells undergo proliferation, changes in cell shape, changes in ion transport, and secrete signaling molecules (Dyer & Cepko, 2000; Reichenbach et al., 2009; Sueishi et al., 1996). Although there are benefits of gliosis as an immune response to injury, there is also the "dark side" of glia (Miller, 2005).

1.2.2 The Goldilocks Paradox of Neuroinflammation

The problem with neuroinflammation and glial reactivity arises when there is too little or too much activation. As a process of gliosis, astrocytes and microglia produce a glial scar that is important in blocking off areas of damage and prevent further damage to the CNS (M. T. Fitch, Doller, Combs, Landreth, & Silver, 1999; Myer, Gurkoff, Lee, Hovda, & Sofroniew, 2006). Not only is this a mechanical obstruction that prevents axon regeneration but inhibitory molecules and cells also play a role (M. Fitch & Silver, 2001; Windle & Chambers, 1950). Glial scaring prevents neuronal recovery in 3 ways:

1. Reactive astrocytes produce inhibitory extracellular matrix (ECM) that bind to receptors on the axons to prevent axonal growth and may contribute to the retrograde atrophy of the axon back to the cell body (Bradbury & Carter, 2011; Fry, Chagnon, López-Vales, Tremblay, & David, 2010; Shen et al., 2009).
2. The ECM in the glial scar may act to retrain neurotrophic factors and cellular stressors (such as reactive oxygen species) that prevent axonal

sprouting and contribute to the atrophy of the neuronal soma (Bradbury & Carter, 2011; Crespo, Asher, Lin, Rhodes, & Fawcett, 2007) .

3. The inhibitory environment of the glial scar may activate macrophages and microglia into a pro-inflammatory state that prevents axonal growth and increases neurotoxicity (Bradbury & Carter, 2011; Rolls et al., 2006; Rolls et al., 2008).

Studies have examined ways to remove the glial scar in an attempt to make the axonal environment more favorable to regeneration. Many studies use chondroitinase ABC to cleave the inhibitory ECM molecule found in the glial scar called chondroitin sulphate proteoglycans (CSPGs) (Bradbury & Carter, 2011; Bradbury et al., 2002; Busch & Silver, 2007; Galtrey & Fawcett, 2007; Lemons, Howland, & Anderson, 1999; Moon, Asher, Rhodes, & Fawcett, 2001; Stichel & Müller, 1998). Chondroitinase ABC has shown to promote axonal growth and sprouting by:

1. Removing the inhibitory ECM (CSPGs) produced by the astrocytes thereby reducing the receptor mediated inhibition (Bradbury & Carter, 2011; Fry et al., 2010; Shen et al., 2009).
2. Degrading the glial scar which acts to liberate growth factors (BDNF) bound to the inhibitory ECM (Bradbury & Carter, 2011; Crespo et al., 2007; Nandini et al., 2004).
3. Producing chondroitin sulphate disaccharides as a result of the degradation of CSPGs. This may have anti-inflammatory and

neuroprotective effects by putting microglia and macrophages into an alternatively activated (anti-inflammatory) M2 activation state (Bradbury & Carter, 2011; Kigerl et al., 2009; Rolls et al., 2004; Rolls et al., 2006). Modulating mediators of neuroinflammation has been demonstrated to improve recovery and survival of neuronal cells after CNS injury. The innate immune response is important for the elimination of pathogens and clearing of debris in order for the CNS to maintain its integrity and survive (Lehnardt, 2010). The problem arises when the inflammation in the CNS becomes chronic or exaggerated, as it is seen in neurodegeneration (Lehnardt, 2010; Medzhitov, 2010).

Excessive microglial activation is neurotoxic due to the over production of superoxides, nitric oxide (NO), and TNFα (M. L. Block, Zecca, & Hong, 2007; C. A. Colton & Gilbert, 1987; Koeberle & Ball, 1999; Liu et al., 2002; McGuire et al., 2001; Moss & Bates, 2001). Another consequence of poorly regulated neuroinflammation is that it can cause collateral injury due to the accumulation of inflammatory factors and cells that make the microenvironment neurotoxic (Lehnardt, 2010). Normally, collateral damage is reversible in non-CNS organs because of an inherent ability to regenerate, however, it results in irreversible neuronal damage in the CNS due to:

1. Reactive oxygen species produced from the respiratory burst of microglia that make neurons susceptible to cell death (Boje & Arora, 1992; Lehnardt, 2010; Liu & Hong, 2003).

2. Inhibitory ECM produced by microglia and astrocytes do not promote axonal growth and sprouting (Bradbury & Carter, 2011; Bradbury et al., 2002; Goldberg & Barres, 2000; Moon et al., 2001; Shen et al., 2009).
3. Neurons are post-mitotic and there is limited repopulation of lost neurons by neuronal precursor cells (Björklund & Lindvall, 2000).

It is not currently known if neuronal death seen after neuroinflammation causes, or is the result of neurodegeneration. However, it clearly plays a crucial role in CNS injury and degeneration. Neuroinflammation after injury often gets a bad reputation but neuroinflammation plays an important beneficial role as well.

The neuroinflammatory response after CNS injury helps to isolate the damaged area, provides protection from infection, and may be beneficial for the survival of neurons. There is increased neuronal loss, inhibited repair of the blood brain barrier (BBB), and inhibition of remyelination when there is too little activation of astrocytes or total inhibition of astrogliosis (Faulkner et al., 2004). This may be because astrocytes produce neurotrophic factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) after injury (Lucas et al., 2006). Similarly, microglia produce pro-inflammatory substances but are also beneficial to survival of neurons because they remove debris and harmful substances from the CNS (Lucas et al., 2006). Cytokines, such as tumor necrosis factor alpha (TNFα), are known to be cytotoxic to neurons and oligodendrocytes but when TNFα was blocked in a clinical trial, it exacerbated

the degeneration in patients with multiple sclerosis (MS) (Arnason & Group, 1999). This clearly demonstrates that under activation of glial cells after CNS injury is detrimental to the survival of neuronal cells and that in an ideal situation there cannot be too little or too much neuroinflammation. It is currently poorly understood under what conditions neuroinflammation is beneficial or detrimental after injury. The time course and type of injury may determine if the outcome will be neuroprotective or cytotoxic.

1.3 The Innate Immune Cell of the CNS: Microglia

Microglia are the innate immune cells of the CNS and play an important role in maintaining and protecting the integrity of the CNS. Due to the BBB, there is very limited infiltration of systemic immune cells into the CNS, especially in the absence of CNS injury and BBB disruption. The origin of microglial cells is somewhat of a controversy (figure 1.2). The dominate theory was that before the BBB closed, monocytes migrated into the CNS and differentiated into microglia (S. Gordon, 2003). However, more recently it was suggested that microglia are derived from extra-embryonic yolk sac myeloid cells that migrate into the CNS before the BBB closes (Ransohoff & Cardona, 2010). Determining the origin of microglia may be important because there seems to be a functional distinction between blood marrow derived macrophages and microglia (Jung & Schwartz, 2012). When there is inflammation of the CNS, microglial activation is supplemented by transient infiltration of monocyte derived macrophages (Ajami, Bennett, Krieger, McNagny, & Rossi, 2011). Resident microglia are the first to

respond and serve to clean up after injury. Microglial activation may be actively terminated by monocyte derived macrophages that infiltrate from the systemic system, as that is the role of monocyte derived macrophages in non-CNS tissue (S. Gordon & Taylor, 2005). Microglia and monocyte derived macrophages are hard to distinguish from each other and their different origins may help explain the difference in function (Jung & Schwartz, 2012).

Another point of controversy in the field has been whether populations of microglia are replenished by monocytes that differentiate to microglia from the blood or if resident microglia replicate and replenish their own population after the BBB has closed (Hess et al., 2004). As explained above, it is now thought that microglia are not derived from monocytes and that they do not contribute to microglial turnover (Jung & Schwartz, 2012). This was proven by using chimeric mice that have monocytes that express green fluorescent protein (GFP) but microglia that did not. The myelo-ablated mice that over expressed human mutant superoxide dismutase 1 (mSOD) did not have significantly different amount of GFP+ cells in the spinal cord when they were asymptomatic (Solomon et al., 2006). Blood marrow derived cells only migrated into the CNS when there was a disruption in the BBB (Jung & Schwartz, 2012). This demonstrated that microglia population self-renew and monocytes derived macrophages were not involved in the overturn of microglia.

1.3.1 Microglia function in normal tissue

Resting microglia have a ramified morphology and have processes that are highly mobile (Davalos et al., 2005; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Fine filopodia like protrusions are formed and withdrawn suggesting that they are able to scan the environment without damaging neuronal structures (Davalos et al., 2005; U.-K. Hanisch & H. Kettenmann, 2007). Neighboring microglia take turns monitoring shared areas and are able to monitor the whole brain parenchyma within a few hours (Davalos et al., 2005; U.-K. Hanisch & H. Kettenmann, 2007; Haynes et al., 2006). It is this random screening that turns to targeted movement when there is any type of injury or pathogen in the CNS (U.-K. Hanisch & H. Kettenmann, 2007).

Microglial cells act to remove toxic cellular debris and secrete neurotrophic factors after the onset of neurological pathologies (Nakajima et al., 2001). Microglia regulate the production of ciliary neurotrophic factor (CNTF) and insulin-like growth factor 1 (IGF-1) by astrocytes by secreting cytokines, such as IL-1 (Nguyen, Julien, & Rivest, 2002). Microglia also change the expression of neurotrophic factors, such as nerve growth factor (NGF), CNTF, and glial cell-derived neurotrophic factor (GDNF) in photoreceptor cells (Harada et al., 2002). In addition, microglia derived NGF, BDNF, and CNTF may indirectly influence neuronal survival in the eye by modulating basic fibroblast growth factor (bFGF) and GDNF production by Müller cells (Harada et al., 2002). Previous studies have shown that grafting activated microglia directly into a lesioned spinal cord supported neurite growth (Rabchevsky & Streit, 1997).

There is also recent evidence to show that microglia may be important in monitoring, maintaining, and eliminating synapses in the normal CNS (Graeber, 2010; Paolicelli et al., 2011; Stevens et al., 2007; Tremblay, Lowery, & Majewska, 2010). The highly motile processes of microglia often make transient contact with synapses frequently (Davalos et al., 2005; Nimmerjahn et al., 2005; Paolicelli et al., 2011; Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). During synapse maturation, neurons up-regulate expression of chemokine fractalkine (Cx3cl 1) (Mody et al., 2001). The Cx3cl 1 receptor is only found on microglia in the CNS and is crucial for migration of microglia (Cardona et al., 2006; Harrison et al., 1998; Jung et al., 2000; Liang et al., 2009). Presynaptic proteins (such as SNAP25) and postsynaptic proteins (such as PSD95) were found in microglia labeled with GFP during synaptic maturation, demonstrating that microglia engulf pre and postsynaptic synapses in the uninjured brain (Paolicelli et al., 2011). There is also decreased synaptic pruning in Cx3cr1 knockout mice and an increase in immature connectivity demonstrating that microglia are important in maintaining synapse number and function (Paolicelli et al., 2011).

1.3.2 The Good and the Bad Attributes of Microglia

Microglial cells respond to CNS injury or other pathogenic stimuli by transforming from a ramified state to an amoeboid form and express surface proteins such as CD14, major histocompatibility complex (MHC), and chemokine receptors (Cho et al., 2006; Fetler & Amigorena, 2005; Nimmerjahn et al., 2005; Regen et al., 2011; Rock et al., 2004). Activation can act to enhance neuronal

survival by enhancing cellular maintenance (such as helping clear toxic debris and material) and providing innate immunity (Jack et al., 2005; Simard, Soulet, Gowing, Julien, & Rivest, 2006; Streit, 2002; Town, Nikolic, & Tan, 2005). They also can help regulate developmental elimination of neuronal cells and release trophic and anti-inflammatory factors (Dougherty, Dreyfus, & Black, 2000; Marín-Teva et al., 2004; Morgan, Taylor, & Pocock, 2004; Upender & Naegele, 2000). Microglia have also been found to help guide stem cells to the site of injury and inflammation which may aid in repair and neurogenesis (Aarum, Sandberg, Haeberlein, & Persson, 2003; Polazzi & Contestabile, 2002; Walton et al., 2006; Ziv, Avidan, Pluchino, Martino, & Schwartz, 2006).

Although microglia can have many beneficial functions after injury, there are circumstances where microglia are highly detrimental to neuronal survival. They can become over activated and produce excessive amounts of cytotoxic factors such as ROS, nitric oxide, and TNF-α (C. A. Colton & Gilbert, 1987; Lee, Liu, Dickson, Brosnan, & Berman, 1993; Liu et al., 2002). It is unclear what causes microglia to be neuroprotective or neurotoxic after injury. The time course and nature of the injury may be an important factor in defining the roles of microglia. Constant activation of microglia in chronic injuries and neurodegenerative disorders may play an important role in the pathology of these conditions (M. L. Block et al., 2007). It becomes important to determine how to push microglia into a neuroprotective state, prevent them from being cytotoxic, and show what the characteristics of different microglial states are at certain times

of the injury. This is why neuroinflammation and microglial activation in CNS injury and neurodegenerative disorders has become a popular focus of neuroscience research.

1.3.3 Microglial Activation and Regulation

Microglia actively monitor the CNS and have evolved many receptors to recognize molecules to react to (M. L. Block et al., 2007; Ransohoff & Perry, 2009). These pattern recognition receptors (PRRs) and they bind to pathogen associated molecular patterns (PAMPs), and are critical part of the innate immune response (Akira, Uematsu, & Takeuchi, 2006). PRRs are important for the identification of stimuli, the production of superoxides, release of pro-inflammatory molecules, and phagocytosis (M. L. Block et al., 2007; Esen & Kielian, 2006; Saijo & Glass, 2011). Activation of PRRs leads to the production of ROS by NADPH oxidase, production of pro-inflammatory compounds, and phagocytosis of the toxin (figure 1.3) (M. L. Block et al., 2007). Microglia may become neurotoxic when PAMPs cause an excessive immune response or when some stimuli are recognized as a pathogen (M. L. Block et al., 2007). Microglial activation may be neuroprotective or cytotoxic depending on how many PRR combinations are made to the same PAMP leading to a cumulative response by the microglia to the same stimuli (M. L. Block et al., 2007).

Microglial toxicity becomes progressively worse as the time they have been activated progresses, which could be the bases of the progressive nature of neurodegeneration (Huh et al., 2003; McGeer, Schwab, Parent, & Doudet, 2003).

This has been demonstrated using lipopolysaccharide (LPS) to stimulate microglia, where microglial activation over time resulted in the progressive loss of dopamine neurons (Gao et al., 2002; Gibbons & Dragunow, 2006; Ling, Zhu, Snyder, Lipton, & Carvey, 2006; Qin, Liu, Hong, & Crews, 2013). LPS is derived from gram-negative bacteria and is commonly used to elicit a strong inflammatory response (Jang et al., 2007; Zheng et al., 2012). Microglia become neurotoxic and take on the pro-inflammatory phenotype after LPS activation (U. K. Hanisch & H. Kettenmann, 2007; Martinez, Sica, Mantovani, & Locati, 2008). However, repeated low dose injections of LPS can have neuroprotective effects (Z. Chen et al., 2012). The problem with using LPS and other substances to activate microglial cells *in vivo* is that it often activate other cell types, such as astrocytes and Müller cells, and any effects on neurons cannot be attributed to microglial activation alone (Fernandes, Silva, Falcão, Brito, & Brites, 2004; Goureau, Hicks, Courtois, & De Kozak, 2002; Jang et al., 2007; Schumann et al., 1998).

Over activation of microglia by the toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can continue for years after exposure, as demonstrated in primates and humans (Langston et al., 1999; McGeer et al., 2003). Interestingly, MPTP damages the neurons directly and results in the indirect over activation of microglia (McGeer et al., 2003; Wu et al., 2003). However, the toxic effects of MPTP was reduced when pro-inflammatory factors were knocked out, suggesting that microglia become over activated and act to

intensify the inflammatory response leading to bystander cell death (M. L. Block & Hong, 2005; Jackson-Lewis et al., 2002; Wu et al., 2003). These studies demonstrate that microglia can become over activated by direct activation or by indirect activation though injury signals from surrounding cells. This can result in a cycle of death (figure 1.4; reactive microgliosis) where injured neurons over activate microglia that in turn damages nearby cells, resulting in more microglial activation (M. L. Block et al., 2007).

The CNS has endogenous protective features that inhibit the over activation of microglia with the use of cannabinoids, neuropeptides, IL-10, transforming growth factor β (TGF β), and microglial apoptosis (M. Block et al., 2006; Boche, Cunningham, Gauldie, & Perry, 2003; Peng et al., 2005; Rivest, 2006). It is when this system fails that neuronal death may occur contributing to the progression of neurodegenerative diseases (Polazzi & Contestabile, 2002). Intracellular ROS helps regulate microglial activation (figure 1.5) (M. L. Block et al., 2007). NADPH oxidase is important for both extracellular and intracellular ROS and superoxide production (Mander, Jekabsone, & Brown, 2006). Generally, the greater the amount of intracellular ROS, the more inflammatory response there is by the microglia (Qin et al., 2004). Intracellular ROS is a secondary messenger that changes gene expression by downstream kinase cascades and transcription factor activation (M. L. Block et al., 2007). The cell is normally able to keep intracellular ROS levels under control by internal antioxidants (M. L. Block et al., 2007; Innamorato, Lastres-Becker, & Cuadrado, 2009; Possel,

Noack, Keilhoff, & Wolf, 2002). As intracellular ROS increases and antioxidants decrease, ROS amplifies the pro-inflammatory response of the microglia (M. L. Block et al., 2007; Qin et al., 2004). However, when intracellular levels of ROS increase beyond a manageable level and antioxidative defenses fail, the microglia cell goes through apoptosis or necrosis, similar to other immune cells (M. L. Block et al., 2007; Mayadas & Cullere, 2005). This mechanism is thought to exist to stop microglia from becoming neurotoxic, but it is when this mechanism fails that microglia may be prevented from cell death resulting in neuronal damage (M. L. Block et al., 2007; Engelhardt, Sen, & Oberley, 2001; Lull & Block, 2010; Zhao et al., 2011).

1.4 Molecular Changes in Neurons after Neuroinflammation

When microglia become activated, they release molecules that damage neurons, such as reactive oxygen species (ROS), tumor necrosis factor α (TNFα), and nitric oxide (Cao & He, 2013; D. Kim et al., 2010; Koeberle & Ball, 1999; Liao, Zhao, Beers, Henkel, & Appel, 2012; Yoshida, Yoshida, & Ishibashi, 2004) (Horvath, Nutile-McMenemy, Alkaitis, & DeLeo, 2008). Not only is there a change in gene expression in microglia but microglial activation may influence gene expression in neurons. After lipopolysaccharide induced neuroinflammation in the brain, there is decreased expression of ephrin receptor B1 (*EphB1*) in the cortex and hippocampus of the mouse brain (Bonow, Aïd, Zhang, Becker, & Bosetti, 2008). *EphB1* is important in the differentiation and migration of neuronal precursors in the adult hippocampus and in the dynamic changes of

dendritic spines (Chumley, Catchpole, Silvany, Kernie, & Henkemeyer, 2007; Henkemeyer, Itkis, Ngo, Hickmott, & Ethell, 2003).

Another that may change expression after neuroinflammation is BM88. BM88 (C38 or Cend1 for cell cycle exit and neuronal differentiation) is a protein that developmentally induces cell cycle exit and neuronal differentiation. BM88 is an integral membrane protein that contains two 22-24 kDa polypeptide chains that are connected by disulphide bonds (Georgopoulou et al., 2006). It is anchored to the outer membrane of mitochondria and the endoplasmic reticulum but may also exist in the cytoplasm (Georgopoulou et al., 2006). BM88 is expressed by retinal ganglion cells (RGCs) and horizontal cells in the retina (Wakabayashi et al., 2010). BM88 overexpression in cultured cells results in cell cycle arrest and silencing the BM88 gene accelerates cell proliferation (P.K. Politis, D. Thomaidou, & R. Matsas, 2008). BM88 forms a part of the p53- cyclin D1- pRb signalling pathway (see figure 1.6; purple pathway) that leads to an end of the cell cycle at the G_0 phase (P.K. Politis et al., 2008). BM88 induces p53 and p21 which inhibits cyclin D1 function (P.K. Politis et al., 2008). Since cyclin D1 is inhibited, it can no longer phosphorylate pRb leading to cell cycle exit. Another reported role of BM88 in development is related to neuronal differentiation. Late in neurogenesis, BM88 acts on proneural genes, Mash1 and neurogenin1, to cause neuronal progenitors to differentiate into neuronal precursor cells and neurons (P.K. Politis et al., 2008). It also releases Notch inhibition of proneural genes resulting in neuronal differentiation (Katsimpardi et al., 2008).

BM88 also plays a role in calcium buffering and therefore may have a neuroprotective role (see figure 1.6; green pathway). BM88 overexpression in neuroblastoma cells show a neuroprotective effective against many neurotoxic stimuli (P.K. Politis et al., 2008). BM88 inhibits proliferation of the neuron by promoting the activity of the P2Y2 receptor but decreasing the activity of the P2Y1 receptor (Masgrau et al., 2009). How it may achieve this is by blocking the release of calcium from inositol 1,4,5-triphosposte (IP_3) sensitive stores, such as the endoplasmic reticulum, by either acting on IP_3 or the IP_3R (Masgrau et al., 2009). This calcium buffering activity prevents apoptosis by increasing the uptake of calcium by the mitochondria and lowering levels of calcium inside the endoplasmic reticulum (Masgrau et al., 2009). Another anti-apoptotic role for BM88 is its ability to inhibit ERK (Masgrau et al., 2009). When ERK is inhibited, BAX cannot bind to the mitochondria which prevents apoptosis (see figure 1.6: black pathway).

The cell cycle of differentiated neurons must be kept under constant control because re-initiation of the cell cycle can lead to cell death (P.K. Politis et al., 2008). In neurodegenerative diseases, there may be dysregulation of cell cycle arrest that may put neurons into an apoptosis-prone state (P.K. Politis et al., 2008). There are examples of cell cycle protein re-expression in Alzheimer's disease, Parkinson' disease, ischemia, and amyotrophic lateral sclerosis (Bonda et al., 2010; Hoglinger et al., 2007; Nguyen et al., 2003; Rashidian et al., 2005; Vincent, Rosado, & Davies, 1996). The importance of down-regulating cyclin D1

in post-mitotic neurons is emphasized by the observation that cyclin D1 re-expression is observed in neurons from patients with neurodegenerative diseases (Hoozemans et al., 2002). Alzheimer's disease neurons contain higher levels of cell cycle markers, particularly cyclin D, cdk4 and Ki67, demonstrating a departure from the G_0 phase (McShea, Harris, Webster, Wahl, & Smith, 1997; Moh et al., 2011; Ogawa et al., 2003). This may act to put the neurons into an apoptosis prone state (Panagiotis K Politis, Dimitra Thomaidou, & Rebecca Matsas, 2008). Microglial activation and neuroinflammation may play a role in cell cycle dysfunction, as shown by a study that demonstrated that NSAIDs were able to prevent neurons from re-entering the cell cycle in Alzheimer's disease prone mice (Varvel et al., 2009). Hence, BM88 dysregulation may therefore lead to neuronal death through three mechanisms: (1) cell cycle dysregulation, (2) mitochondria dysfunction, and (3) impaired calcium regulation.

1.5 Neuroinflammation as a Target of Disease

Two popular approaches to target neuroinflammation in helping treat a disease or CNS injury are (1) to use neuroinflammatory mechanisms to eliminate harmful by-products of neurodegeneration and (2) to modulate the neuroinflammatory response in a way that makes the environment more neuroprotective.

1.5.1 Harnessing Neuroinflammation for Treatment and Repair

In rodent models of Alzheimer's disease and even in human samples, microglia are attracted to amyloid deposits in the brain (Simard et al., 2006). Bone

marrow derived microglia (BMDM; also called bone marrow derived macrophages or monocyte derived marcophages/microglia) also respond to amyloid plaques and may even act to slow the progression of Alzheimer's disease by removing the plaques from the CNS (Simard et al., 2006). The toll like receptor 2 (TLR2) on microglia recognizes amyloid β and when it was knocked out in an mouse model of Alzheimer's disease, there was a greater decline in spatial and contextual memory (Richard, Filali, Préfontaine, & Rivest, 2008). However, there was a restoration of the cognitive decline when TLR2 was rescued in the bone marrow cells of the knockout mice (Richard et al., 2008). In addition, BMDM are put into an alternative, neuroprotective activation state when TGF β and Smad 2/3 (downstream signaling of TGF β) were blocked, increasing amyloid β clearance and improving Alzheimer's like pathology (Town et al., 2008).

Patients with presymptomatic Alzheimer's disease or mild cognitive impairment have low levels of macrophage colony stimulating factor (M-CSF) and low levels of hematopoietic cytokines which predicts rapid progress to dementia in 2-6 years (Ray et al., 2007). M-CSF is responsible for differentiating hematopoietic stem cells into macrophages, as well as promoting phagocytosis and chemotaxis (Stanley et al., 1997). Administration of M-CSF into the systemic system activated BMDM to degrade amyloid β and improve the cognitive decline seen in mouse models of Alzheimer's disease (Boissonneault et al., 2009). Targeting microglia, BMDM, and hematopoietic precursor cells and their

neuroinflammatory mechanisms are good candidates for future immune system therapies for neurodegenerative disorders and CNS injuries.

1.5.2 Modulating Neuroinflammation

Microglia exist on a spectrum of activation states that range from the pro-inflammatory (M1) state to the neuroprotective (M2) state (Henkel, Beers, Zhao, & Appel, 2009). M1 microglia produce substances that damage neurons, such as tumor necrosis factor α (TNFα), nitric oxide, interleukin 1β (IL-1β), and reactive oxygen species (ROS) (Cao & He, 2013; David & Kroner, 2011; Liao et al., 2012). M2 microglia express substances that are neuroprotective, including IL-4, IL-10, arignase1, BDNF, and other neurotrophic factors (Bessis, Béchade, Bernard, & Roumier, 2007; David & Kroner, 2011; Liao et al., 2012). Experimentally, LPS can be used to promote an M1 phenotype, while IL-4 is known to promote an M2 phenotype (Fenn, Henry, Huang, Dugan, & Godbout, 2012; Michelucci, Heurtaux, Grandbarbe, Morga, & Heuschling, 2009). The balance between these two cell types may determine if microglia are more beneficial or detrimental after injury and may play a role in neurodegenerative diseases (C. Colton et al., 2006; Lucin & Wyss-Coray, 2009).

The question becomes if it is possible to stimulate the microglia to be neuroprotective and promote survival and regeneration, while inhibiting them from becoming pro-inflammatory. Leukocytes and microglia are able to produce anti-inflammatory cytokines and neurotrophic factors that are known to be neuroprotective (Yong & Rivest, 2009). A drug for MS, glatiramer acetate, has

been demonstrated to increase the production of anti-inflammatory cytokines and neurotrophic factors leading to greater remyelination after spinal cord injury (Skihar et al., 2009). This clearly demonstrates that putting immune cells in an alternative activation state can lead to neuroprotective outcomes and suggest that these pathways are good candidates for therapeutic intervention.

Another method of modulating neuroinflammation is to inhibit the activation of immune cells. Minocycline is a tetracycline derivative that can cross the BBB and is often used to inhibit microglia activation and transmigration of T lymphocytes in experimental models of neurodegenerative diseases and CNS injury (H.-S. Kim & Suh, 2009; Stirling, Koochesfahani, Steeves, & Tetzlaff, 2005; Wasserman, Zhu, & Schlichter, 2007). It was originally used for its antimicrobial properties but is now being investigated for its anti-inflammatory properties in ischemia, traumatic brain injury, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, and multiple sclerosis (M. Chen et al., 2000; Choi et al., 2007; Jackson-Lewis et al., 2002; Luccarini et al., 2008; Mejia, Ona, Li, & Friedlander, 2001; Yong et al., 2004; Yrjänheikki et al., 1999; Zhu et al., 2002). Minocycline modulates neuroinflammation by reducing proliferation and activation of microglial cells and reducing the production of cytokines, chemokines, lipids, matrix metalloproteinases (MMPs), nitric oxide release, superoxide generation, and neutrophil chemotaxis (Dommergues, Plaisant, Verney, & Gressens, 2003; He, Appel, & Le, 2001; Horvath et al., 2008; H.-S. Kim & Suh, 2009; Tikka,

Fiebich, Goldsteins, Keinänen, & Koistinaho, 2001; Wasserman et al., 2007). Minocycline is thought to inhibit microglial activation, but a recent study showed that minocycline may selectively inhibit microglia from taking on the M1 phenotype but not the M2 phenotype (Kobayashi et al., 2013).

There is a lot of interest around using minocycline for the treatment of neurodegenerative diseases and there have been human clinical trials for using it as part of the treatment for schizophrenia, Alzheimer's disease, spinal cord injury, and stroke (Casha et al., 2012; Chaudhry et al., 2012; Mangialasche, Solomon, Winblad, Mecocci, & Kivipelto, 2010; Switzer et al., 2011). Minocycline reduces BBB damage, edema, and neurological deficits after intracerebral hemorrhage in rats (Power et al., 2003; Wasserman & Schlichter, 2007). However, there have been other data to suggest that minocycline may not be as neuroprotective as previously thought. A phase III randomized trial showed that ALS patients had faster declines in the Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) and greater mortality than the placebo groups when given minocycline (P. H. Gordon et al., 2007).

1.6 Conclusion

The field of immunology is one riddled with countless debates, particularly when it comes to neuroinflammation. In 1884, Elias Metchnikoff described an amoeboid cell from starfish larvae that engulfed and digested cellular debris leading him to conclude that there are inflammatory cells that are phagocytic and have beneficial functions (Kaufmann, 2008). While others

claimed that these phagocytic cells are the major players in inflammatory diseases (Tauber, 1992). Even the long held notion that the CNS is totally immune privileged has been challenged following numerous studies demonstrating an interaction between the immune system and the CNS (Carson, Doose, Melchior, Schmid, & Ploix, 2006). There is also controversy over whether microglia play a neuroprotective or cytotoxic role after injury or neurodegeneration (Rock et al., 2004). It is clear that neuroinflammation plays a role in CNS injuries and diseases. It is important to understand the role that microglia and other immune cells play in the CNS to be able to develop safe treatments. The time course, type of injury, and the type of treatment option that is used may determine if the outcome is neuroprotective or cytotoxic. Studying the mechanisms and finer details of neuroinflammation can help provide new therapeutic targets for therapies.

1.7 Figures and Tables

Table 1.1: The main features of neuroinflammation. Inflammation in the CNS is different from other tissues in the body. The most obvious difference is the delayed and very little recruitment of systemic immune cells, such as leukocytes. Regardless of the differences, there are some features of inflammation that are the same in the CNS and rest of the body. Adapted from (Lucas et al., 2006).

Table 1: Main Features of Neuroinflammation
Oedema
MHC expression
System acute phase response
Acute phase protein synthesis
Glial activation
Complement activation
Synthesis of inflammatory mediators (cytokines and ROS)
Expression of adhesion molecules
Invasion of immune cells

Figure 1.1: The archetype of the inflammatory pathway. The inflammatory process involves inducers, sensors, mediators, and the tissue. Inducers are the stimuli that begin the inflammatory response when detected by the sensors. Sensors are receptors (such as the TLR) that are expressed on immune cells, such as macrophages, dendritic cells, microglia, and mast cells. These cells produce the mediators that effect the target tissue in attempt to resolve the noxious conditions (Medzhitov, 2010).

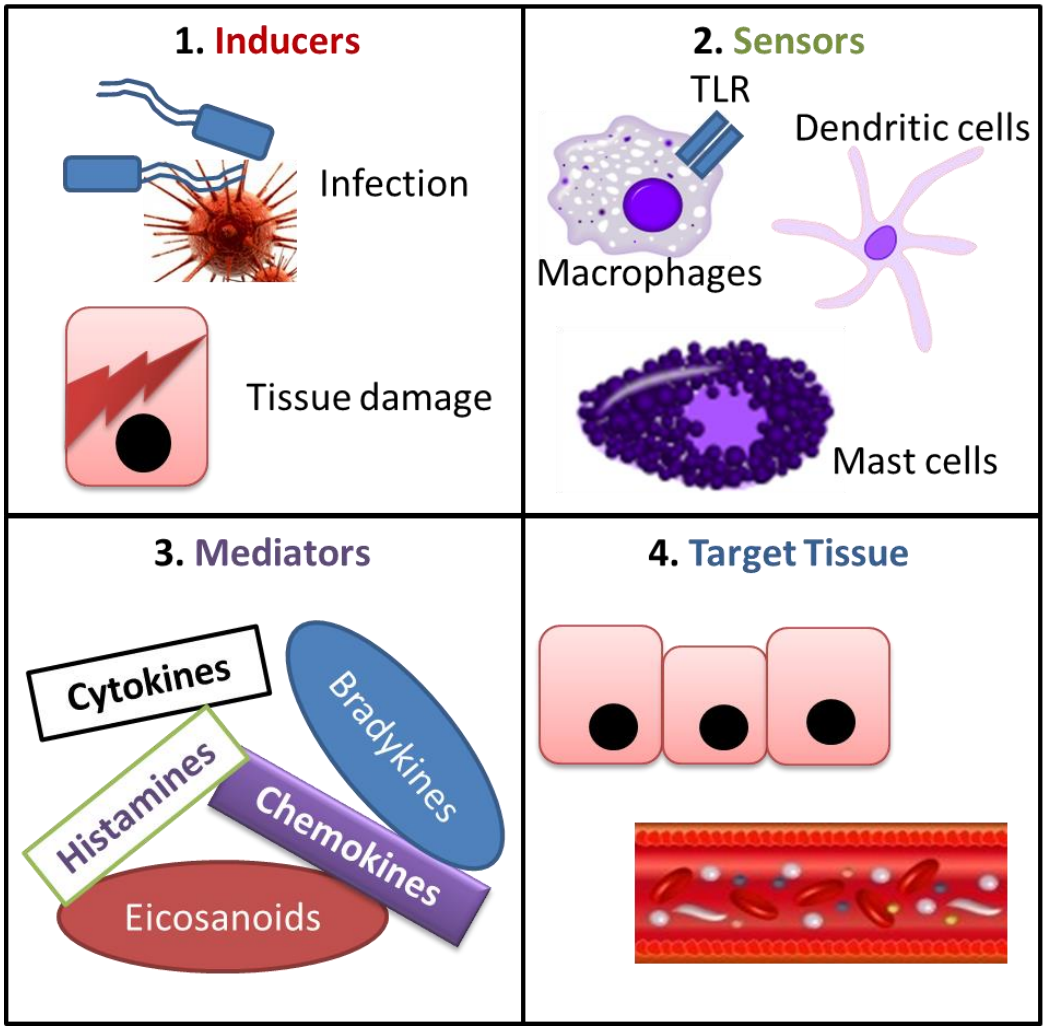

1. Inducers
Infection
Tissue damage
2. Sensors
TLR
Dendritic cells
Macrophages
Mast cells
3. Mediators
Cytokines
Bradykines
Histamines
Chemokines
Eicosanoids
4. Target Tissue

Figure 1.2: The two leading theories for microglial lineage. (A) Some studies suggest that microglia are derived from monocytes that cross the BBB before it closes (it has also been questioned if monocytes are able to cross the BBB after it is formed and differentiate into microglia). (B) Others suggest that microglia are derived from extra embryonic yolk sac myeloid cells that populate the CNS before the BBB closed. This theory suggest that microglia are not from the same lineage as monocyte derived macrophages and, therefore, may explain their functional difference. Both theories agree that microglia are derived from a hematopoietic stem cell and not from neuroectodermal progenitors that macroglia and neurons are derived from.

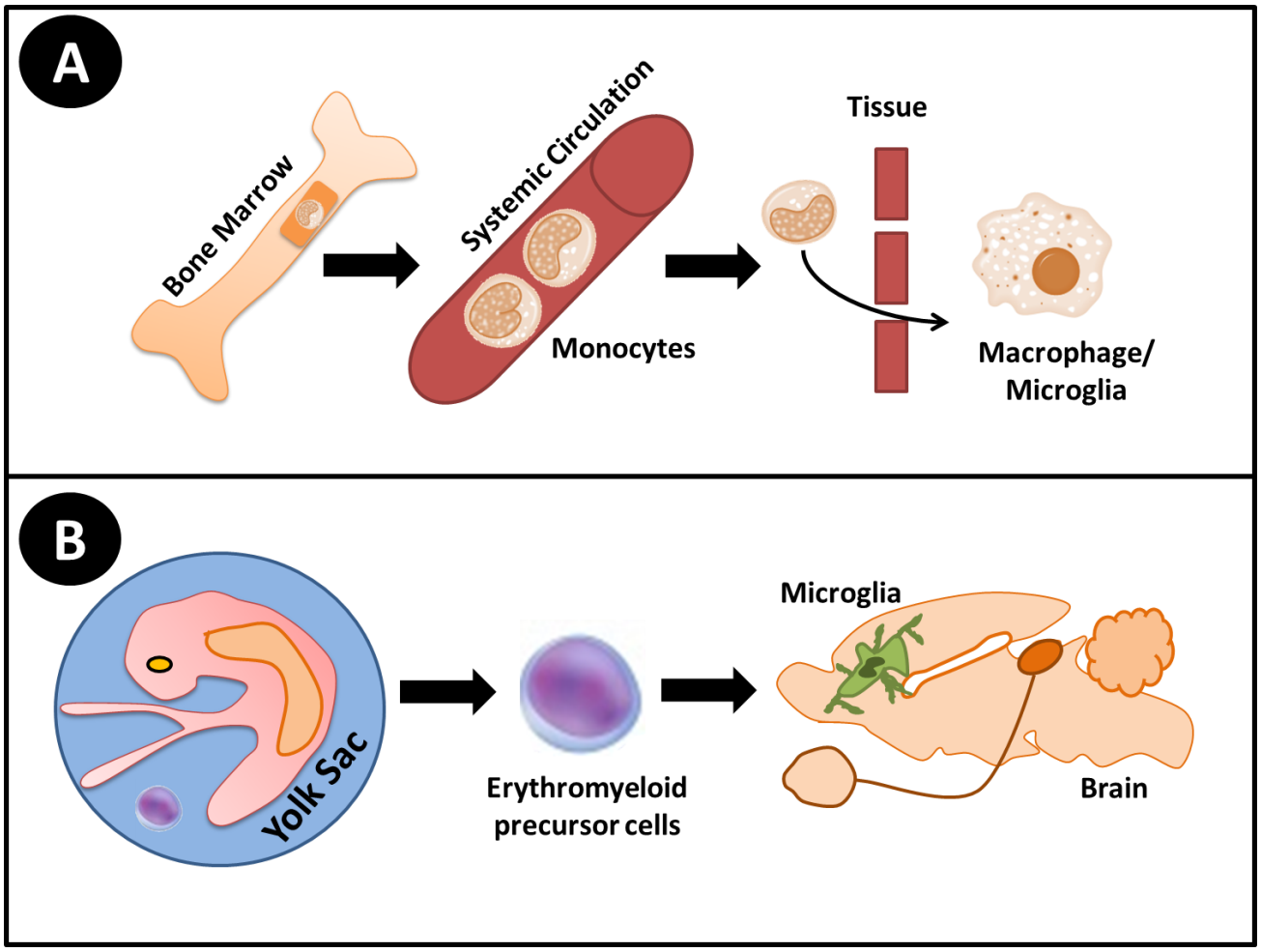

A
Bone Marrow
Systemic Circulation
Monocytes
Tissue
Macrophage/
Microglia
B
Yolk Sac
Erythromyeloid
precursor cells
Microglia
Brain

Figure 1.3: Pattern recognition receptors (PRRs) on microglia bind to pathogen associated molecular patterns (PAMPs) to initiate the inflammatory response. Microglia actively explore the CNS for stressors using PRRs. Once activated, they are responsible for recognizing the pathogen, producing ROS, releasing cytokines, and destroying the toxic stimuli by phagocytosis. This process can become neurotoxic if the response to PAMPs is excessive or loses its regulation.

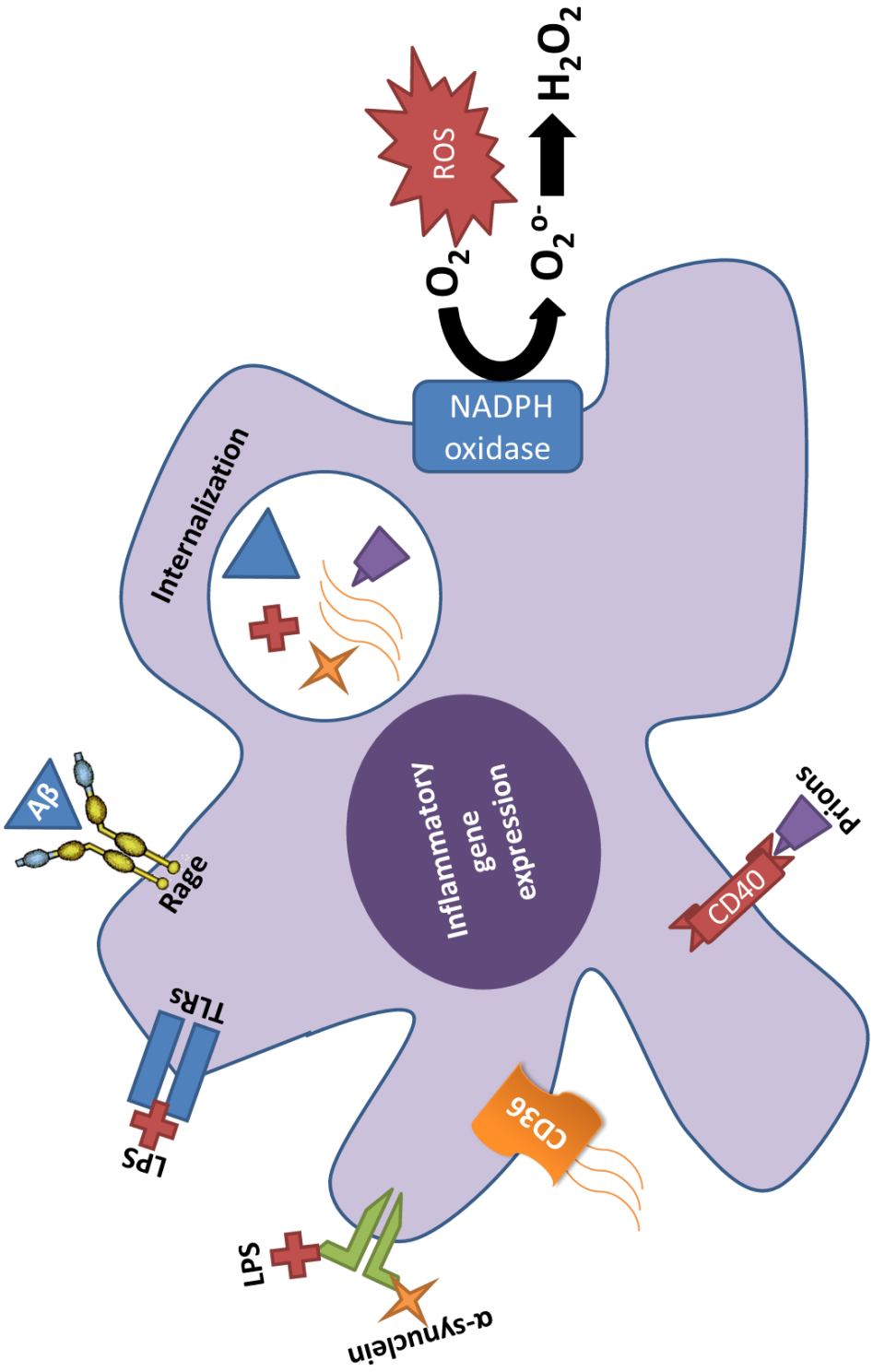

ROS
H_2O_2
O_2^{o-}
O_2
NADPH oxidase
Internalization
Inflammatory gene expression
Aβ
Rage
TLRs
LPS
LPS
α-synuclein
CD36
CD40
Prions

Figure 1.4: Neurotoxicity due to reactive microgliosis. There are two ways that microglia can become over activated and serve to be cytotoxic; (1) some stimulus activates the microglia to a pro-inflammatory state that damages the neuron or (2) neuronal damage leads to microglia becoming over activated and neurotoxic. This leads to a repeating cycle of death because the over activation of microglia causes damage to surrounding neurons which over activates other microglia.

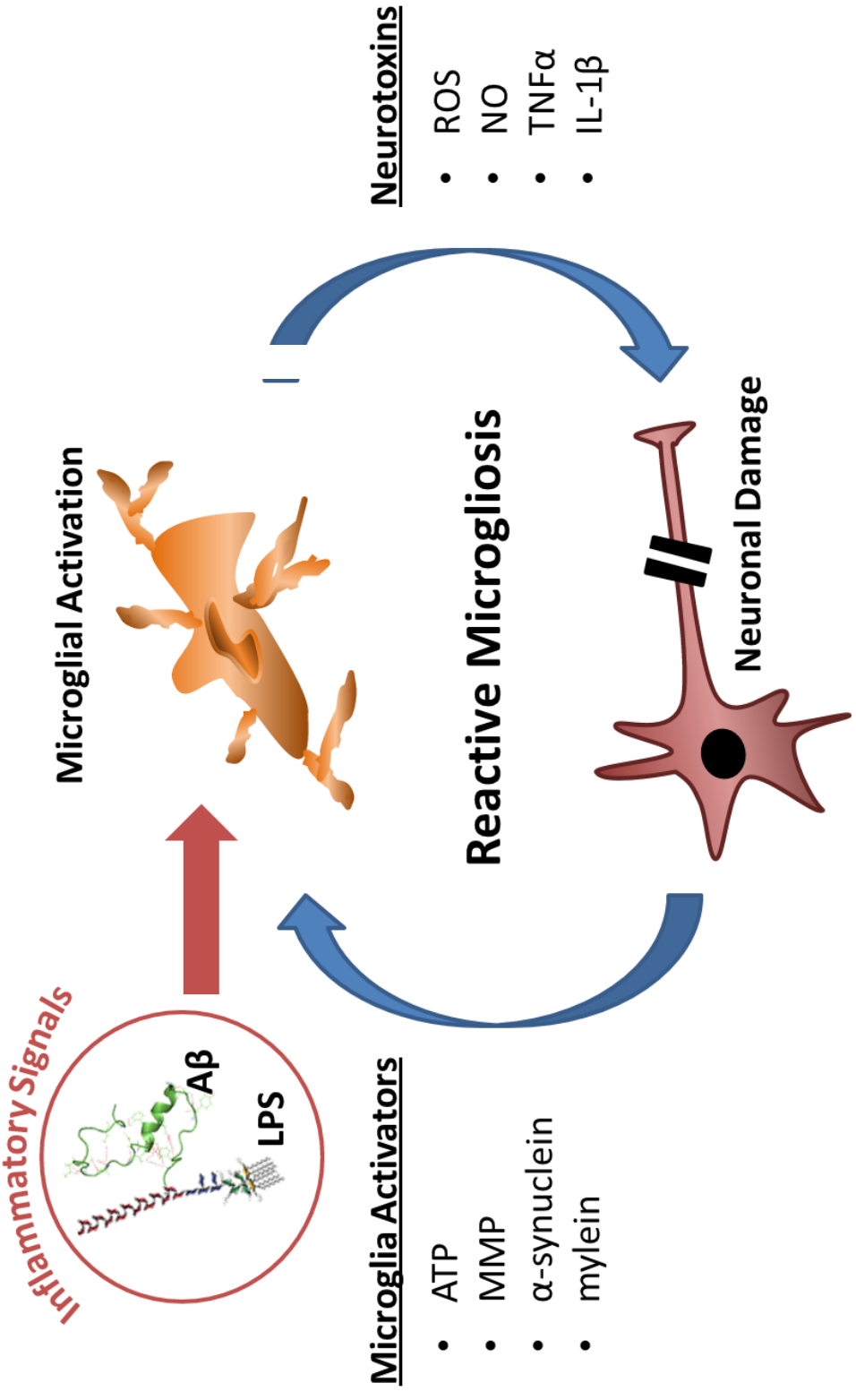

Inflammatory Signals
Aβ
LPS
Microglia Activators
• ATP
• MMP
• α-synuclein
• mylein
Microglial Activation
Reactive Microgliosis
Neurotoxins
• ROS
• NO
• TNFα
• IL-1β
Neuronal Damage

Figure 1.5: Microglial activation is regulated by intracellular ROS levels. ROS inside of microglia acts as a secondary messenger that determines the inflammatory function of the cell and its survival. Normally microglia have low levels of intracellular ROS that are removed by the cells antioxidants. In response to pathogens, internal levels of ROS increases allowing the cell to respond to the stimuli. As a protective mechanism for neuronal cells, when the intracellular level of ROS becomes too great, the microglial cell goes through cell death. However, microglia may become over activated when these internal mechanisms fail resulting in a neurotoxic outcome.

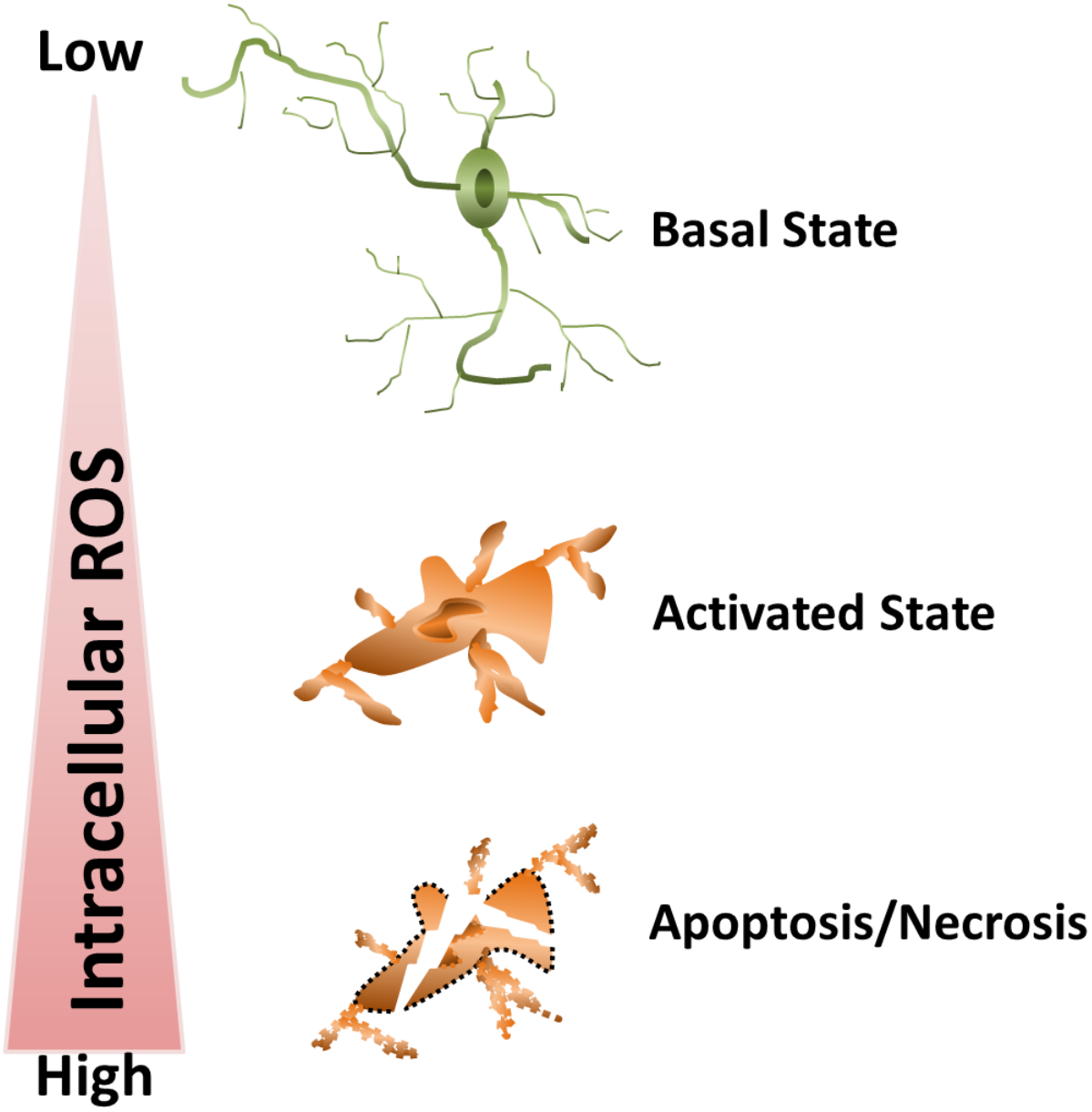

Low
Intracellular ROS
High
Basal State
Activated State
Apoptosis/Necrosis

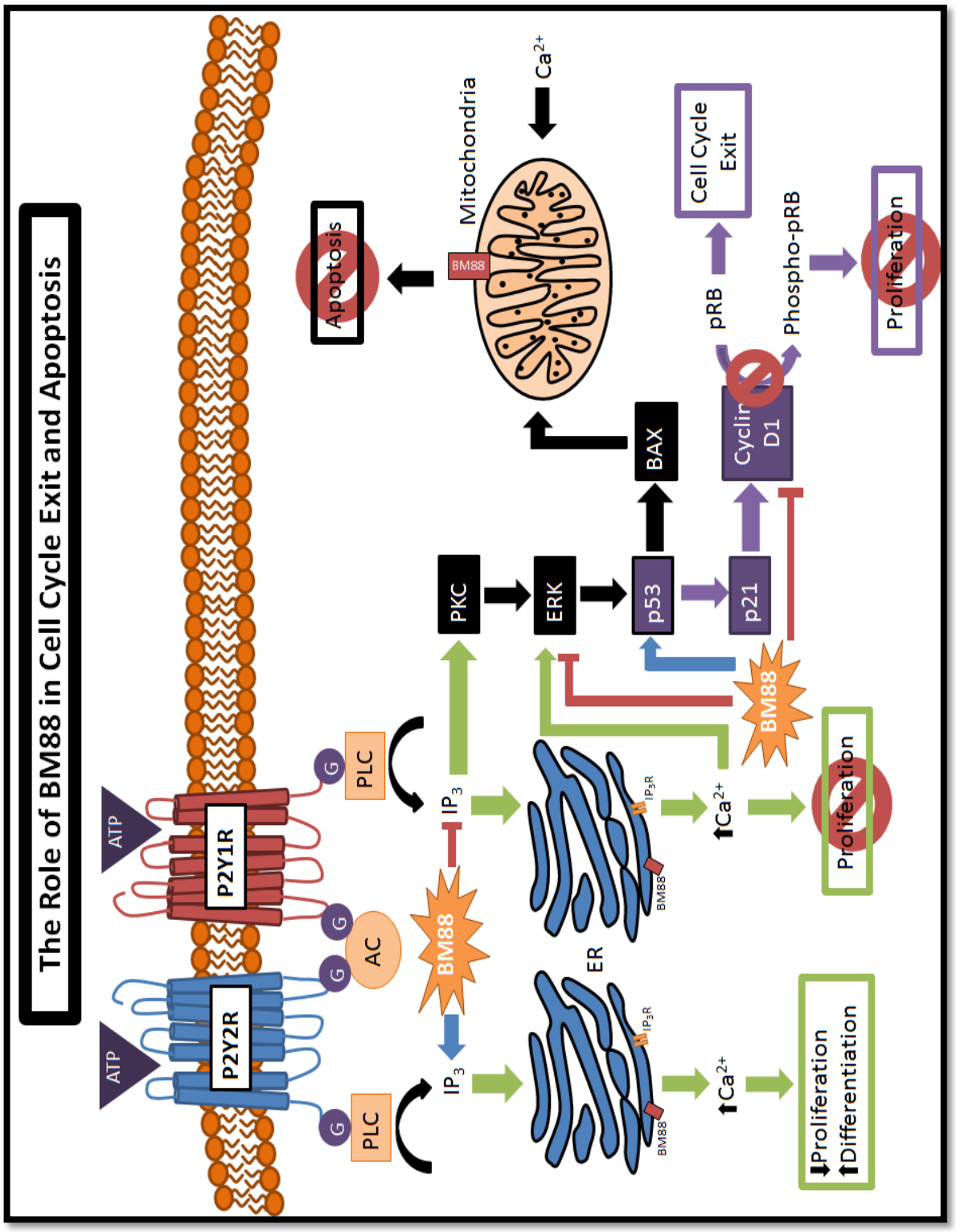
Figure 1.6: The role of BM88 in cell cycle exit and preventing apoptosis.

There are 3 functions BM88 plays in order to prevent the cell from undergoing apoptosis and becoming post mitotic:

(1) BM88 promotes P2Y2R and inhibits P2Y1R. Both lead to release of calcium from the endoplasmic reticulum (ER) which decreases proliferation (green pathway).

(2) Calcium in the cytosol would normally activate ERK and IP3 would activate PKC and by extension ERK. ERK acts on p53 and BAX downstream. When BAX binds the mitochondria this leads to apoptosis of the cell due to mitochondrial dysfunction. BM88 acts to inhibit ERK and prevents apoptosis of the cell (black pathway).

(3) The p53-p21-cyclin D1 pathway is important in maintaining proliferation of the cell. BM88 acts to prevent cyclin D1 from phosphorylating pRB leading to cell cycle exit (purple pathway).


The Role of BM88 in Cell Cycle Exit and Apoptosis
ATP
P2Y1R
ATP
P2Y2R
G
PLC
G
AC
G
PLC
IP3
IP3
BM88
BM88
ER
IP3R
IP3R
BM88
BM88
Ca2+
Ca2+
PKC
ERK
p53
p21
BAX
Cyclin D1
pRB
Phospho-pRB
Cell Cycle Exit
Proliferation
Proliferation
Mitochondria
Ca2+
BM88
Apoptosis
Proliferation
Differentiation

CHAPTER 2: RATIONAL AND HYPOTHESIS

2.1 Glaucoma and the Role of Neuroinflammation

In the evolution of humans, vision has become the dominant sensory modality and its loss has profound effects on survival and quality of life. The RGCs (RGCs) are the only neurons that send visual information from the retina to the brain so their death results in irreversible blindness. The mechanisms that lead to the death of RGCs after injury are not well understood but several suspected causes have been proposed. These include neurotrophic factor deprivation, ischemia, reactive astrocytosis, microglial over activity, mitochondrial dysfunction and excitotoxicity, to name a few (Almasieh, Wilson, Morquette, Cueva Vargas, & Di Polo, 2012; Hernandez, 2000; Howell et al., 2011; T. V. Johnson, Bull, & Martin, 2011; Kong, Van Bergen, Trounce, & Crowston, 2009; Nguyen et al., 2011; H. Quigley, 1999; Sun et al., 2010). Similar to other central nervous system (CNS) neurons, RGCs have a limited ability to survive or regenerate after injury (Lebrun-Julien & Di Polo, 2008). Therefore, RGCs are critical for visual perception and the mechanisms that govern their structural and functional integrity are important to study.

2.2 Glaucoma

In the mammalian eye, the retina is composed of layers of cells that absorbs light and coverts it to an electrical signal (figure 2.1) (E. Dreyer & Lipton, 1999). The visual signal undergoes processing in the synaptic plexiform layers that reach the innermost retinal layer consisting of RGCs (E. Dreyer & Lipton, 1999). The axons of the RGCs form the optic nerve which exits the eye at

the lamina cribrosa and primarily terminates in the lateral geniculate nucleus and superior colliculus in humans (E. Dreyer & Lipton, 1999).

Glaucoma is described as a group of optic neuropathies that results in the slow degeneration of RGCs and their axons (R. Weinreb & Khaw, 2004). Clinical signs of glaucoma include increased cupping of the optic nerve and visual field loss leading to blindness (Mikelberg et al., 1995; Pena et al., 2001). The changes and degeneration are not only limited to RGCs but trans-synaptic loss of neurons in the lateral geniculate nucleus and visual cortex have been reported as well (Gupta, Ang, de Tilly, Bidaisee, & Yücel, 2006). In addition, retinal glial cells are activated and may lead to the death of RGCs by altering the environment in which the axons reside (Pena et al., 2001; Wang, Cioffi, Cull, Dong, & Fortune, 2002).

It is estimated that 60.5 million people worldwide will have glaucoma in 2010, rising to 79.6 million by 2020 (H. Quigley & Broman, 2006). Glaucoma is currently the second leading cause of blindness in the world and it disproportionately affects women and Asians (H. Quigley & Broman, 2006). Glaucoma is categorized as either Open (OAG) or Closed Angle (CAG), depending on geometry of the aqueous humor outflow apparatus located at the base of the iris. 74% of people with glaucoma will have the OAG subtype and women will comprise 59% of all glaucoma patients in 2010 (H. Quigley & Broman, 2006). The Asian population represents 47% of those affected and 87% of the people affected by CAG (H. Quigley & Broman, 2006). Since most cases of glaucoma are of the OAG variety, there is no obvious cause for a rise in

intraocular pressure (IOP). Furthermore, glaucoma may occur without a significant elevation in IOP. Although the progression of the disease is slowed by lowering IOP, even when IOP is not elevated, elevated IOP is considered to be a risk factor (Quigley, 2005), and not the cause of glaucoma. In the past, OAG was divided into subgroups as high tension (HTG) or normal tension (NTG) depending on the IOP (Ariani et al., 2006). Patients with NTG experience RGC loss and have shown to benefit from lowering of IOP. Therefore, raised IOP can only be considered a risk factor for OAG (Ekström, 2012; H. A. Quigley, 2005). It has also been documented that the percentage of patients with NTG varies according to the population (H. A. Quigley, 2005), which may indicated a genetic component to glaucoma.

2.2.1 Possible Causes for Glaucoma – Classical Theories

In 1858, there were 2 theories presented by Müller and von Jaeger (independently) to explain optic nerve damage resulting from glaucoma (E. Dreyer & Lipton, 1999). Müller believed that RGC death was caused by mechanical trauma to the optic nerve from raised intraocular pressure (IOP) in the eye (E. Dreyer & Lipton, 1999). However, von Jaeger believed that the vascular system was compromised in the eye leading to glaucoma (E. Dreyer & Lipton, 1999). Both theories still have strong proponents and the debate continues. In the 1970s, however, it was proposed that raised IOP would lead to a blockage of axoplasmic flow at the lamina cribrosa (Anderson & Hendrickson, 1974; Lampert, Vogel, & Zimmerman, 1968). This blockage may prevent neurotrophins

from reaching the soma of RGCs triggering apoptosis (E. Dreyer & Lipton, 1999; Harry A Quigley et al., 2000).

Glaucoma has generally been thought to involve elevated IOP due to impaired aqueous outflow from the anterior chamber of the eye (Epstein, Allingham, & Schuman, 1997; Gabelt & Kaufman, 2005). Surgical therapies can lower IOP but 25% to 38% of individuals still develop visual field loss (E. Dreyer & Lipton, 1999). These observations have led to the conclusion that there must be other factors other than raised IOP that lead to the development of glaucoma.

2.2.2 Possible Causes for Glaucoma – Current Theories

The current dominate theory has become that glaucoma is a result of damage to the RGCs due to damage to the optic nerve head after raised IOP (figure 2.2) (Crish, Sappington, Inman, Horner, & Calkins, 2010; Howell et al., 2007; Lebrun-Julien & Di Polo, 2008). The optic nerve head has been implicated because the sectorial pattern of visual field loss highly correlated to the areas that were damaged (H. Quigley, Katz, Derick, Gilbert, & Sommer, 1992). High IOP should be thought of more as a risk factor for glaucoma and not the main cause because RGC death is seen under normal IOP (Qu, Wang, & Grosskreutz, 2010). Since the mechanisms involved in glaucoma are not well known, IOP has been the only modifiable factor and most treatments aim to lower IOP (Y. H. Kwon, Fingert, Kuehn, & Alward, 2009).

It is starting to become more evident that glaucoma probably has more than one factor that contributes to RGC death and that treatment should take a

multifaceted approach. RGCs in glaucoma die through the process of apoptosis, which can be due to, but not limited to, neurotrophin deprivation, ischemia, excitotoxicity, oxidative stress, and glial activation (Qu et al., 2010). Many glaucoma models, such as axotomy/optic nerve crush, microbead occlusion model, genetic models (DBA/2J), and the Morrison model, mimic RGC death due to the stressors described above (Garcia-Valenzulela, Gorczyca, Darzynkiewicz, & Sharma, 1994; Kalesnykas et al., 2012; Morrison et al., 1997; Harry A Quigley et al., 1995; Zhong et al., 2007). These mechanisms are not mutually exclusive and these mediators of RGC death can occur together. However, the end result is that RGCs die by apoptosis and treatment options need to target these different stressors to prevent or delay RGC death.

2.3 Neurotrophic Factor Withdrawal

Neurotrophic withdrawal can cause RGCs to go through apoptosis. Growth factors are required for the continued survival of RGCs. Growth factors are produced by other cells in the vicinity of the neuron's axon terminals (Rabacchi, Ensini, Bonfanti, Gravina, & Maffei, 1994). These target derived growth factors (TDGFs) are transported back to the neuron's cell body where they activate survival pathways (Lebrun-Julien & Di Polo, 2008).

Brain-derived neurotrophic factor (BDNF) signalling is vital for the development and survival of RGCs (figure 2.3) (Fan, Agarwal, & Cooper, 2006). The delivery of BDNF has been shown to rescue RGCs from degeneration (Koeberle & Ball, 2002; Mansour-Robaey, Clarke, Wang, Bray, & Aguayo,

1994). The survival of RGCs may depend on the proper functioning of the intracellular trafficking mechanism for growth factors because they are essential in delivering the survival signal (Butowt & von Bartheld, 2001; Fan et al., 2006). One important gene involved in the intracellular trafficking of BDNF is optineurin. Genetic analysis show that 13.6% of individuals with sporadic glaucoma and 16.7% of individuals with OAG have a mutation in the optineurin (optic neuropathy inducing protein) gene (Vittitow & Borrás, 2002). Optineurin has shown to be important in membrane trafficking and cellular morphogenesis by interacting with Huntingtin and Rab8, linking myosin IV to the Golgi complex, and effecting metabotropic glutamate receptor sensitivity (De Marco, Buono, Troise, & Diez-Roux, 2006; Dhami & Ferguson, 2006; Sahlender et al., 2005),. This pathway becomes important for BDNF transmission because BDNF has been demonstrated to be dependent on the myosin protein complex (Yano et al., 2006).

2.4 Excitotoxicity

A major excitatory neurotransmitter in the retina is glutamate (Qu et al., 2010). The glutamate receptor, N-Methyl-D-Aspartate (NMDA), allows an influx of calcium from the extracellular space into the cell (Qu et al., 2010). Normally, low intracellular calcium levels are maintained by calcium buffering proteins, the mitochondria, and the endoplasmic reticulum (Zündorf & Reiser, 2011). Excessive activation of the glutamate receptor or failure of calcium buffering capabilities would result in the accumulation of the calcium in the cytosol and leading to the activation of apoptosis pathways (Mattson, 2000;

Orrenius, Zhivotovsky, & Nicotera, 2003; Zündorf & Reiser, 2011). *In vivo* and *in vitro* studies have shown that RGCs are susceptible to excitotoxicity and elevated levels of glutamate are found in glaucoma patients (Caprioli, Kitano, & Morgan, 1996; E. B. Dreyer, Zurakowski, Schumer, Podos, & Lipton, 1996; Nguyen et al., 2011). The results from studies demonstrating glutamate excitotoxicity are highly debated due to alleged scientific fraud, however there has been a recent interest in the area of research (Y. Kwon et al., 2004; Lotery, 2005; Massoll, Mando, & Chintala, 2013; Nguyen et al., 2011; Salt & Cordeiro, 2005).

2.5 Oxidative Stress

The brain consumes a large amount of the body's oxygen and derives its energy from oxidative metabolism by the mitochondrial respiratory chain (Coyle & Puttfarcken, 1993). Oxidative stress occurs when cytotoxic oxygen radicals are over produced and the body struggles to remove them (Coyle & Puttfarcken, 1993; Finkel & Holbrook, 2000). NADPH oxidase induces oxidative stress, particularly by reactive oxygen species (ROS) that are generated when microglia become activated (Gao, Zhou, & Hong, 2012). Oxidative stress can result in neuronal damage due to mitochondrial impairment, protein oxidation and aggregation, reduced protein degradation due to dysfunction of the ubiquitin proteasome system (UPS), oxidation of DNA, RNA, and lipids, and autophagy (Gao et al., 2012). Excessive exposure to ROS and oxidative stress can lead to apoptosis of the neuron (Gao et al., 2012).

Oxidative damage has been demonstrated in many neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and macular degeneration (Büeler, 2009; Jarrett & Boulton, 2012; Ohia, Opere, & LeDay, 2005; Padurariu et al., 2010). There is increasing support for the idea that oxidative stress plays a role in glaucoma (Ferreira et al., 2010; Ghanem, Arafa, & El-Baz, 2010; Izzotti, Bagnis, & Sacc 2006; Tezel, 2011). The oxidative stress hypothesis is attractive because it helps account for the late onset of neurodegenerative diseases and their progressive nature (Coyle & Puttfarcken, 1993).

2.6 Neuroinflammation and Glial reactivity

Neuroinflammation and oxidative stress go hand in hand because glia are a main source of oxidative stress after CNS injury (Reynolds, Laurie, Lee Mosley, & Gendelman, 2007). In addition, ROS are able to activate glial cells which attract T-cells and causes them to proliferate and produce TNFα (Tezel et al., 2007). There is growing evidence to suggest that immune system signalling is involved in the pathogenesis of glaucoma (Schwartz, 2007; Tezel, 2011; Tezel & Wax, 2003, 2004). The immune response from T-cells seems to initially limit neurodegeneration, however, after expansion and secondary recruitment T-cells start producing autoantibodies (Gramlich et al., 2012; Kipnis et al., 2002; Wax, Tezel, & Edward, 1998; Yang, Patil, Yu, Gordon, & Wax, 2001). Glaucoma patients have been shown to have an elevated antibody titer against heat shock protein 60 (HSP60) and HSP27 and there were IgG autoantibodies (against

myelin basic protein and optic nerve antigens) detected in the sera (Grus, Joachim, Hoffmann, & Pfeiffer, 2004; S. C. Joachim, Reichelt, Berneiser, Pfeiffer, & Grus, 2008; Wax et al., 1998). Furthermore, when animals are immunized with HSP60, there was a loss of RGCs (S. Joachim, Wax, Seidel, Pfeiffer, & Grus, 2010; S. C. Joachim et al., 2008; Wax et al., 2008). It is currently unclear if immune reactivity is a consequence of, or a cause of the disease.

2.6.1 Gliosis in Glaucoma

Glial activation in glaucoma differs in the retina and optic nerve head (E. C. Johnson & Morrison, 2009). After experimental IOP elevation there is up-regulation of glial fibrillary acidic protein (GFAP) in astrocytes and Müller cells, however, at the optic nerve head there is increased expression of extracellular matrix (ECM) proteins (Hernandez, 2000; E. C. Johnson, Deppmeier, Wentzien, Hsu, & Morrison, 2000; Steele, Inman, Calkins, Horner, & Vetter, 2006). Astrocytes can produce proMMP-9 but require factors from microglia to convert it to the active form (Rosenberg, 2002). Matrix metalloproteinases (MMP) are a group of zinc-dependent endopeptidases that are important in ECM remodelling and neuroinflammation. MMPs catalyze the proteolysis of many ECM proteins such as collagens, laminin, glycoproteins, and proteoglycans (Lemaître & D'Armiento, 2006; Milward, Fitzsimmons, Szklarczyk, & Conant, 2007). This interaction demonstrates that immune and glial cells are important modulators of the ECM. The modulation of the ECM by MMPs is involved in altering the

migratory capabilities T cells and natural killer cells (Kitson et al., 1998; Leppert, Waubant, Galardy, Bunnett, & Hauser, 1995) and allows white blood cells to cross the blood-brain-barrier (BBB) (Leppert et al., 1995). MMP-9 can also disrupt the BBB, so inhibiting MMP-9 prevents BBB permeabilization by immune cells (Mun-Bryce & Rosenberg, 1998; Rosenberg, 1995). Breakdown of the ECM can contribute to neuronal damage by increasing the permeability of blood vessels or by causing a break in communication channels for signalling molecules and nutrients by loosening the connection between cells (Rosenberg, 2002). Cell death can also be caused by an accumulation of ECM with fibrosis that could cause abnormalities in transport of nutrients and trophic factors to the cells (Rosenberg, 2002). Later in the disease, the axonal debris is cleaned up and replaced with a GFAP positive glial scar (E. C. Johnson et al., 2000).

Optic nerve head astrocytes have increased levels of major histocompatibility complex (MHC) antigens in glaucoma (E. C. Johnson & Morrison, 2009). MHC class II are able to activate T-cells, suggesting that optic nerve head astrocytes may function as antigen presenting cells that evoke an immune response (Tezel et al., 2007; Yang, Yang, et al., 2001).

2.6.2 Microglial Activation

In glaucoma, there is activation of astrocytes and microglia which may lead to the death of RGCs by altering the environment in which the axons reside (figure 2.4) (Son et al., 2010; R. Weinreb & Khaw, 2004). Microglia normally function to scavenge toxic agents, supply growth factors, and supply metabolites,

but in response to degeneration will proliferate and migrate to the affected area (Graeber, 2010; Neumann, Kotter, & Franklin, 2009; Santos et al., 2010; Tezel & Wax, 2004). Activated microglial cells also exhibit immunophenotypic changes and release products with either neurotrophic or neurotoxic effects (Langmann, 2007; Wolfgang J Streit, 2002). In response to injury, microglia may initially be beneficial but become harmful later in the injury after becoming over activated.

There are two mechanisms by which microglia can become over activated and have neurotoxic effects. Microglia can recognize pro-inflammatory stimuli (such as lipopolysaccharides) and become over activated causing neuronal damage by producing neurotoxic factors (Block, Zecca, & Hong, 2007). Microglia can also become over activated due to neuronal damage which becomes toxic to neighbouring cells (Block et al., 2007). This cycle of perpetuating death is known as reactive microgliosis and could be a possible mechanism common to many neurodegenerative disorders regardless of the initiating stimuli (Block et al., 2007; Tansey & Goldberg, 2010).

Activated microglia may add to the oxidative stress of the tissue by over producing reactive oxygen species and nitric oxides (Carter & Dick, 2003; W. J. Streit, 1993). This can lead to increased levels of nitric oxide synthase (NOS) which results in the breakdown of the blood retinal barrier leading to an increase of leukostasis, as demonstrated in models of Diabetic Retinopathy (Leal et al., 2007). Activated microglia may also recruit monocytes to the CNS by increasing expression of monocyte chemoattractant protein 1 (MCP-1) (D'Mello, Le, &

Swain, 2009). Some studies show that inhibition of microglial activation can slow degeneration of retinal neurons in axotomized eyes (Thanos & Naskar, 2004), however, other studies show that microglia activation after the onset of many neurological pathologies can be beneficial to surviving cells (Nakajima et al., 2001).

Experimental data shows that inhibition of microglia by minocycline is neuroprotective after injury and disease. In the DBJ/2A mouse model of glaucoma there was reduced microglial activation and improved RGC axonal transport and decreased RGC axon loss (Bosco et al., 2008). Bosco *et al.* (2008) suggested that this was not due to changes in astrocyte or Müller cell gliosis but because there was an increased proportion of resting microglial cells. However, other studies show that any neuroprotective or cytotoxic effects of minocycline do not have to depend on the modulation of microglial cells. Minocycline has been shown to induce the activation of retinal pigmented epithelial cells through the activation of p38 and ERK1/2 (Hollborn, Wiedemann, Bringmann, & Kohen, 2010). Minocycline at higher doses was able to induce the degeneration of the retinal pigmented epithelium (Hollborn et al., 2010). It is also known to inhibit MMP-9 and migration of T lymphocytes, which has shown to be neuroprotective (Yong, Power, Forsyth, & Edwards, 2001). These conflicting results suggest that minocycline is neuroprotective or cytotoxic has also become a topic of debate after a clinical trial showed that ALS patients had faster declines in the Amyotrophic Lateral Sclerosis Functional Rating Scale

(ALSFRS-R) and greater mortality after minocycline treatment than the placebo groups (Gordon et al., 2007).

Optic nerve head microglia are not as well studied as optic nerve head astrocytes. They are highly associated with the vasculature in the optic nerve head and are found near the peripapillary choroid in glaucoma (Lam, Kwong, & Tso, 2003; A. H. Neufeld, 1999). Optic nerve head microglia increase expression of pro-inflammatory TNFα, TGFβ2, and MMPs after activation (Yuan & Neufeld, 2000, 2001). A correlated up-regulation of ionized calcium binding adapter molecule 1 (Iba1; microglia activation marker) by microglia has been shown as the axon degenerates (Imai & Kohsaka, 2002; E. C. Johnson, Jia, Cepurna, Doser, & Morrison, 2007). Retinal microglia act similarly to other microglia in the CNS. They normally reside at the margins of the inner plexiform layer but after experimental glaucoma are activated and will proliferate and migrate to the injury site (Bodeutsch & Thanos, 2000; Inman & Horner, 2007) .

There is evidence that there is increased level of nitric oxide synthase (NOS-1, NOS-2, NOS-3) in the optic nerve head of patients with OAG (A. Neufeld, Hernandez, & Gonzalez, 1997). Increased levels of NOS-1 and NOS-2 suggests that the optic nerve head is exposed to high levels of nitric oxide (NO) (Lipton et al., 1993). NO may interact with superoxide anions and be toxic to RGC axons (Lipton et al., 1993). Activated microglia within the retina are a source of nitric oxide and, therefore, may play a role in glaucoma (W. J. Streit, 1993). The effect of NO on RGC death may lead to primary cell death by causing

injury to axons at the optic nerve head, and secondary cell death by causing injury to RGC somas due to local glial responses surrounding injured ganglion cells. The exact role of microglia after injury is not well understood, particularly whether or not their activation is dualistic in nature. It is important to study the action of microglial cells after injury and determine the conditions in which microglial cells are neurotoxic or neuroprotective in order to find the best therapeutic targets.

2.7 Purpose, Hypothesis, and Significance of this Thesis

It is thought that there is no single population of harmful microglia but that both harmful and beneficial microglial phenotypes co-exist (Gomes-Leal, 2012). What may determine if microglia have neurotoxic or neuroprotective effects after injury may depend on the time course and nature of the injury, as well as the anatomical location where microglia act on. Neuroprotective therapies and mechanisms act to slow or prevent death of neurons, such as RGCs and their axons, to maintain their function (R. N. Weinreb & Levin, 1999). Early in neurodegeneration, microglia may try to initiate repair mechanisms and remodel the tissue, however, after prolonged activation there may be chronic inflammation that leads to neuronal loss (Hanisch & Kettenmann, 2007; Langmann, 2007; Muzio, Martino, & Furlan, 2007; Schuetz & Thanos, 2004). Studying the phenotypic, morphological, and functional changes in microglia after injury is important because they can help expose signaling events that determine their activation (Karlstetter, Ebert, & Langmann, 2010). Due to the diverse phenotypes

of microglia and macrophages, there may be multiple possible targets for therapeutic interventions.

The function of resting retinal microglia and over activated microglia after neuronal injury is not well known. It is my **HYPOTHESIS** that manipulating the activation of microglia to acquire either a pro-inflammatory or pro-survival phenotype will exacerbate neuronal cell death or enhance neuronal survival after injury, respectively. This thesis will explore the fundamental processes that determine microglial activation and demonstrate how different activation states affect neuronal cells located in its environment. This thesis will also reveal under what conditions cultured microglia are able to migrate to the injury site when they are located in the eye or systemic circulatory system. This thesis also provides insight into conditions and time course that cause microglia to assume a beneficial or detrimental phenotype and how they react to different activation stimuli. Ultimately, this research will contribute to the basic sciences by providing a greater understanding of the role of microglial cells and the immune system in neuronal survival and the mechanisms that lead to cell death. Future applications of this work can lead to therapeutic targets and a mechanism by which the effect of drugs on microglia can be studied.

2.8 Figures and Tables

Figure 2.1: Anatomy of the eye and retina. (A) The light enters the eye through the pupil and is detected by the retina at the back of the eye. The photoreceptors that detect the light are at the back of the eye and the light must transverse all the layers of the retina first. The RGCs transmit the visual information from the retina to the brain through the optic nerve. The vitreous is a clear gel substance that fills the space between the lens and retina. (B) Transverse section of a rat retina with all the nuclei labeled with DAPI. The ganglion cell layer (GCL) is composed of RGCs and displaced amacrine cells. The inner nuclear layer (INL) is compared of bipolar cells, amacrine cells, and horizontal cells. The inner plexiform layer (IPL) consists of the synapses between the RGCs and cells of the INL. The outer nuclear layer (ONL) contains the cell bodies of the rods and cones. The retinal pigmented epithelium (RPE) contains melanin and helps decrease the amount of light that scatters. It also helps with the regeneration of the photopigment and maintains the choroid and retina.

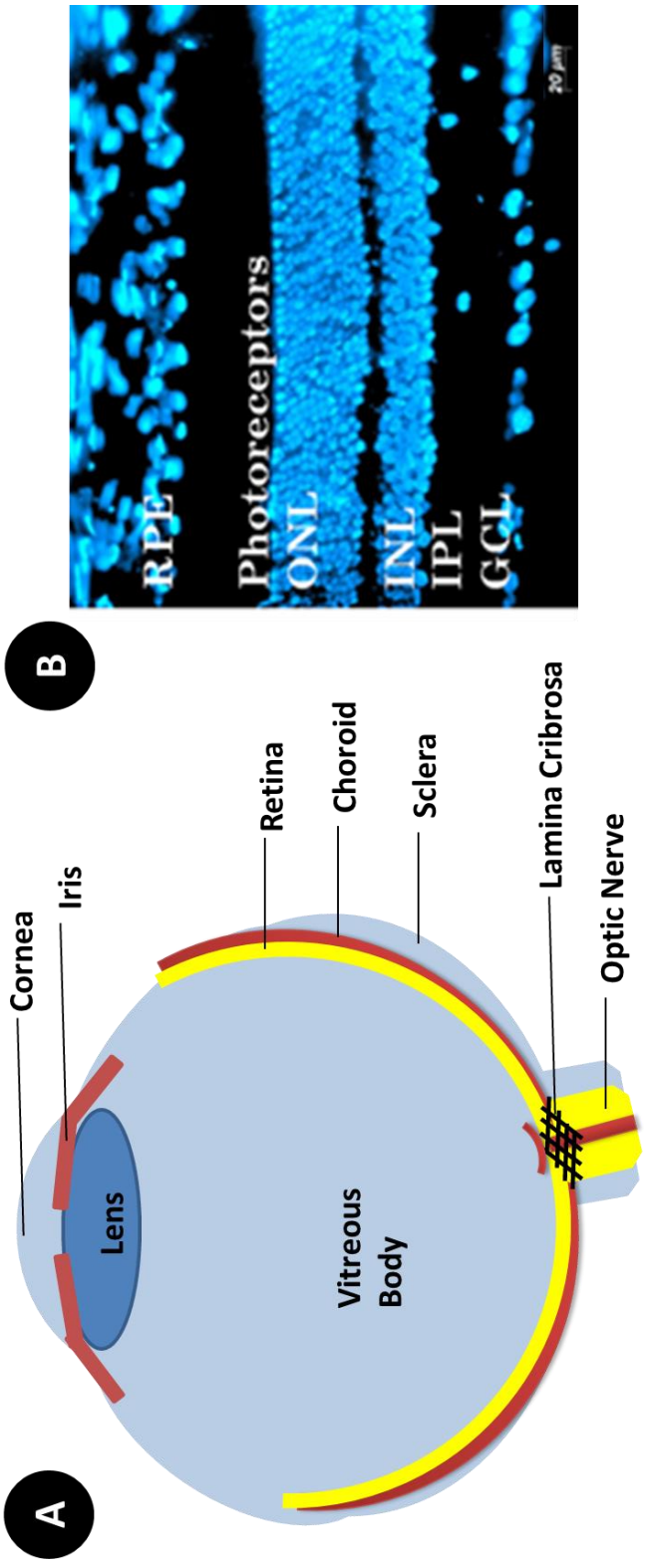

B
RPE
Photoreceptors
ONL
INL
IPL
GCL
A
Cornea
Iris
Retina
Choroid
Sclera
Lamina Cribrosa
Optic Nerve
Lens
Vitreous
Body

Figure 2.2: The dominate theory in glaucoma. The dominate theory in glaucoma is that RGCs die when there is an increase in IOP. (A) IOP may increase due to the slowing of the drainage of aqueous humor from the eye. In open angle glaucoma, the trabecular meshwork becomes blocked. In angle closure glaucoma, the angle between the cornea and iris narrows leading to slow drainage. (B) The axons of the RGCs leave the eye through a mesh like structure, the lamina cribrosa, and form the optic nerve. High IOP causes a distortion of the meshwork leading to narrowing of the holes causing the axons to become impinged. (C) When the optic nerve becomes impinged, this slows down the transport of neurotrophic factors from the target to the soma. When the RGCs are deprived of neurotrophic factors, the cell goes through apoptosis.

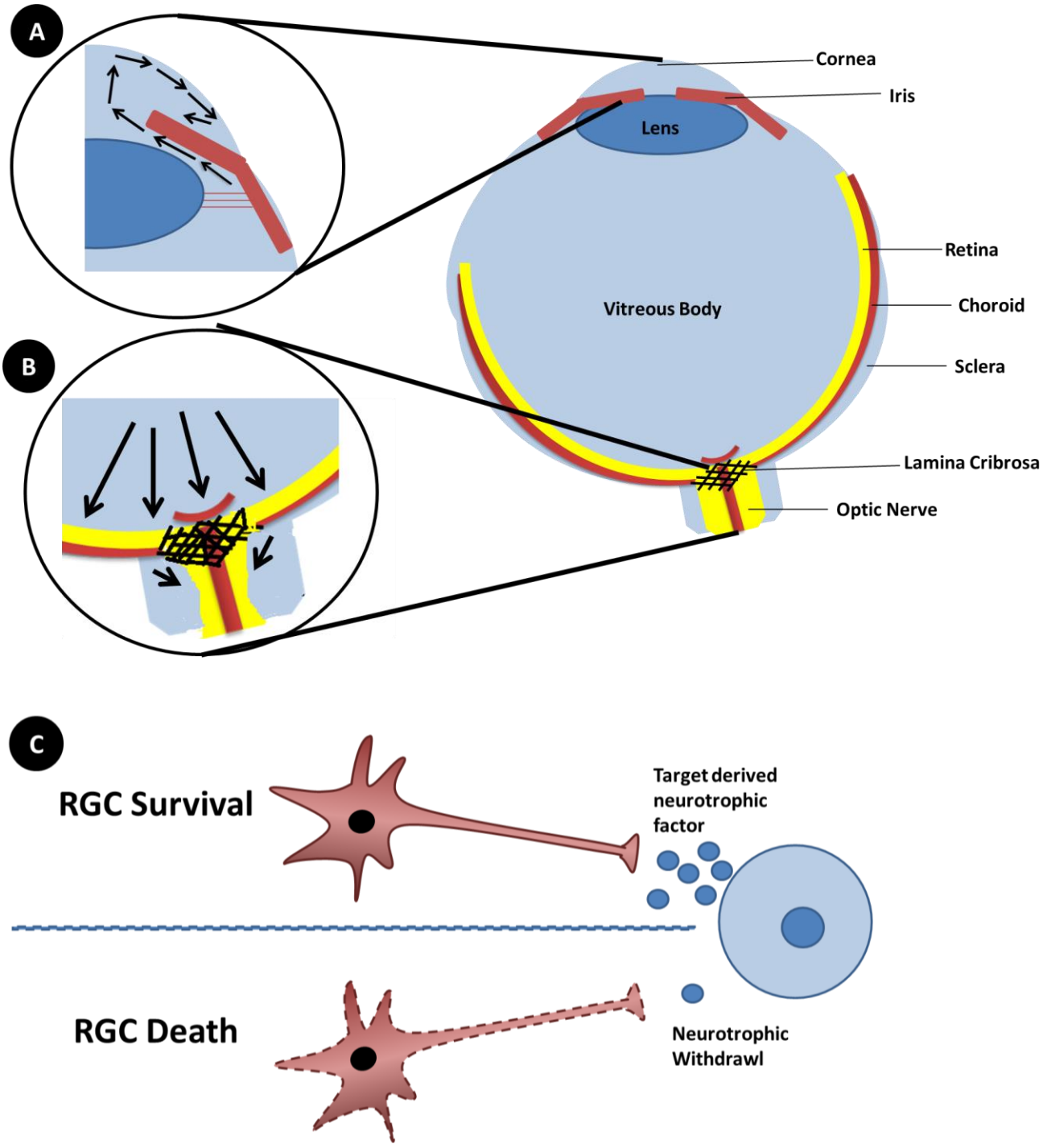

A
B
C
Cornea
Iris
Lens
Retina
Vitreous Body
Choroid
Sclera
Lamina Cribrosa
Optic Nerve
RGC Survival
Target derived
neurotrophic
factor
RGC Death
Neurotrophic
Withdrawl

Figure 2.3: Neurotrophic factor (BDNF) deprivation leads to RGC apoptosis. BDNF normally binds to the Tyrosine kinase receptor B (TrkB) that through an internal cascade promotes the survival of the cell by activating pro survival genes and inactivating BAD (Bcl2 associated death promoter). When BDNF cannot bind TrkB, then BAD is activated and prevents anti-apoptotic proteins from working. This leads to an increase in ROS and activation of apoptosis through caspases.

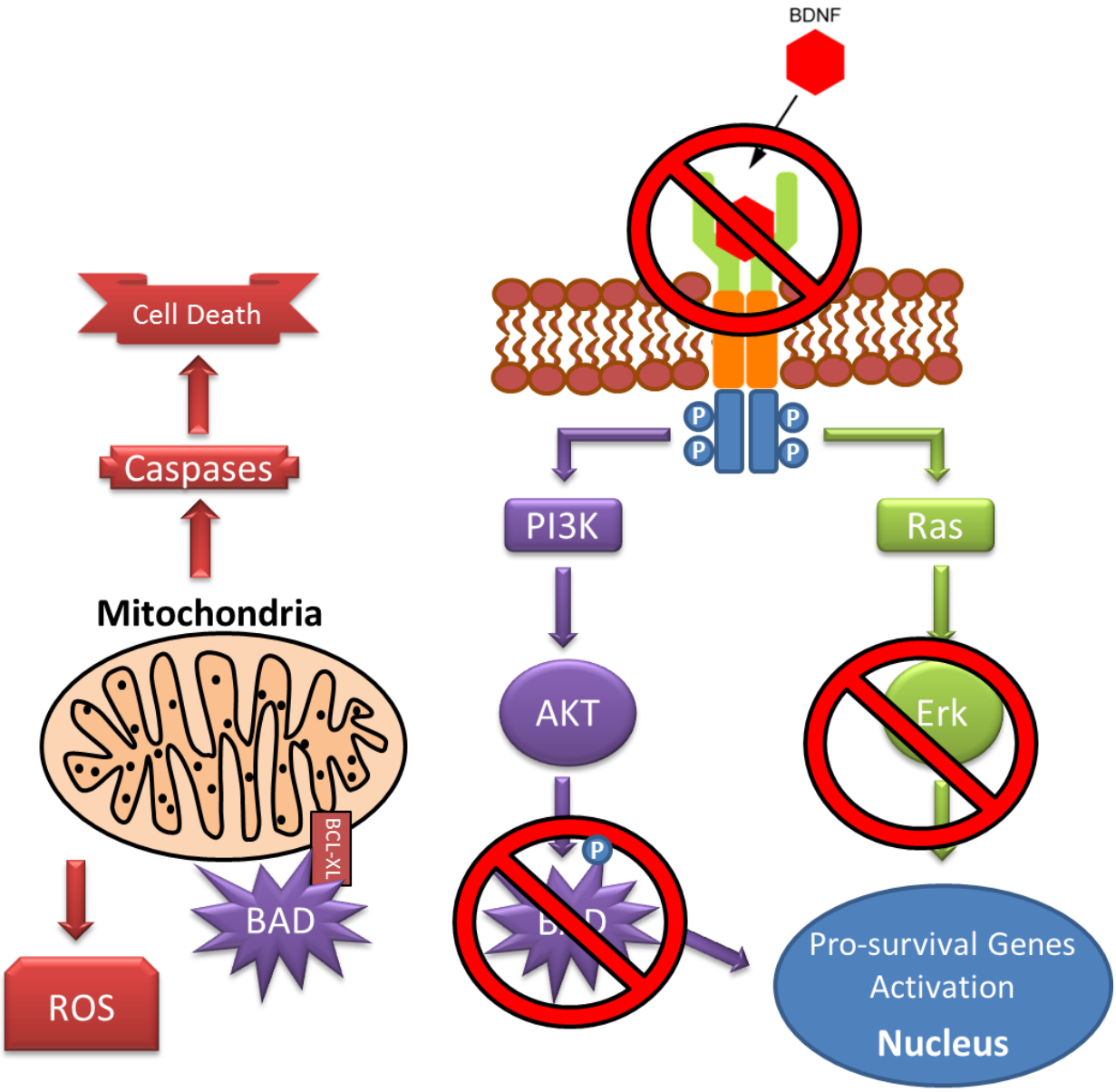
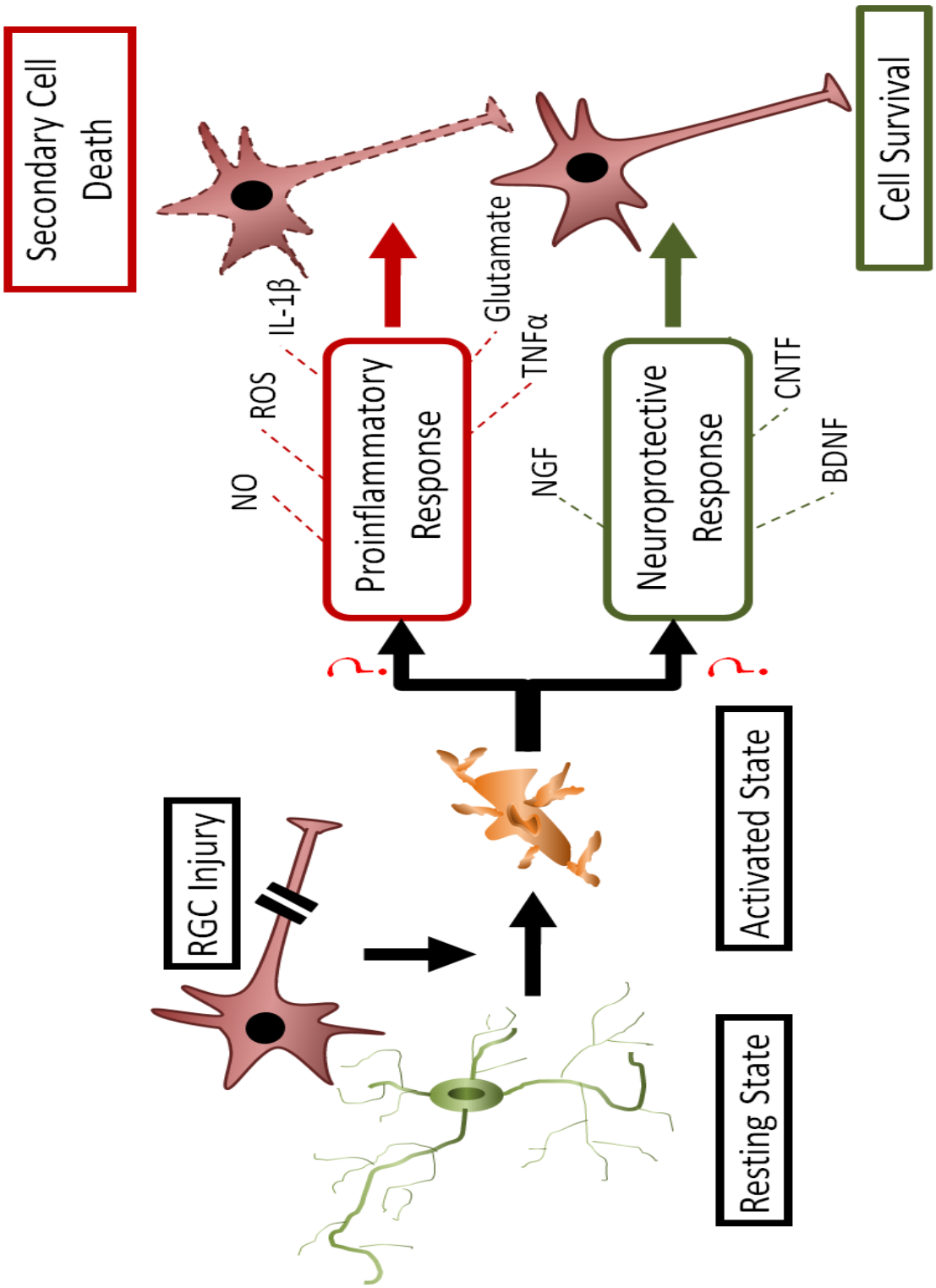

BDNF
Cell Death
Caspases
Mitochondria
BCL-XL
BAD
ROS
PI3K
AKT
Ras
Erk
Pro-survival Genes
Activation
Nucleus

Figure 2.4: Microglia activation after neuronal injury. After RGC injury, microglia become activated where they go from a resting state to an activated state. Activation of microglia results in molecular changes and morphological changes, such as up-regulation of cytokines and going from round cell body with long processes to a more amoeboid shape. What factors determine if activated microglia have a pro-inflammatory or neuroprotective phenotype is currently poorly understood. The pro-inflammatory response is associated with the release of substances such as nitric oxide (NO), reactive oxygen species (ROS), glutamate, interleukins and tumor necrosis factor α (TNFα). This response is thought to result in the death of neurons and collateral damage. The neuroprotective response involves the release of neurotrophic factors such as BDNF, nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF).


Secondary Cell Death
Cell Survival
IL-1β
ROS
NO
Glutamate
TNFα
NGF
CNTF
BDNF
Proinflammatory Response
Neuroprotective Response
?
?
RGC Injury
Activated State
Resting State

CHAPTER 3: EXPERIMENTAL PROTOCOLS

3.1 Cell Culture

HAPI (highly aggressive proliferating immortalized microglial cells) cells are a spontaneously occurring immortalized microglia cell line isolated from neonatal rat brains (Cheepsunthorn, Radov, Menzies, Reid, & Connor, 2001). HAPI cells have morphological similarities to other microglia/macrophages and are able to phagocytose (Cheepsunthorn et al., 2001). HAPI cells also express complement 3 receptors, similar to microglia and brain macrophages, as well as glucose transport protein 5, which is exclusively found on microglia (Cheepsunthorn et al., 2001; Payne, Maher, Simpson, Mattice, & Davies, 1997). Activation of HAPI cells with lipopolysaccharides (LPS) induces mRNA and secretion of tumor necrosis factor α (TNFα) and nitric oxides (NO), similar to primary microglia (Cheepsunthorn et al., 2001).

HAPI (gifted Dr. J.R. Connor) or rMC-1 cells (control; immortalized retinal Müller cell line 1; gifted by Dr. V.P. Sarthy) were cultured in Dulbecco's modified Eagle's medium – F12 (DMEM-F12; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich; St. Louis, MO), 100 U/mL penicillin/100 μg/mL streptomycin (Gibco, Grand Island, NY). The cells were incubated at 37^{o}C in humidified 5% CO2. The cells were passaged 1:10 after they had reached 90% confluency. Culture media was changed every 3 days.

3.2 Labelling and Imaging of HAPI and rMC-1 Cells

HAPI or rMC-1 cells were grown on round coverslips in a 24 well plate. The cells were grown until they were 80% confluent and were washed in wheat

germ agglutinin diluted in PBS for 10 minutes. HAPI cells were labelled with wheat germ agglutinin conjugated (WGA) to Oregon Green (OG) fluor and the rMC-1 cells were labelled with wheat germ agglutinin conjugated to Texas Red (TR) fluor. The wheat germ agglutinin was then removed and normal growth media was placed in the wells after 3 washes with PBS. Every day after the labelling, a coverslip containing cells was washed in phosphate buffered saline (PBS; 0.01M, pH 7.3) and fixed in 4% paraformaldehyde, 2% sucrose in Sorensen's phosphate buffer (0.1M, pH 7.3) for 10 minutes. The cells are then washed in PBS and the coverslip was inverted on to a glass slide containing Vectashield with DAPI stain (Vectorlab). The cells are then visualized under a fluorescent microscope.

3.3 Immunohistochemistry of Cultured Cells

HAPI and rMC-1 cells were grown on round coverslips in a 24 well plate separately. The cells were grown until they were 80% confluent. A coverslip containing cells was washed in PBS and fixed in 4% paraformaldehyde, 2% sucrose in Sorensen's phosphate buffer (0.1M, pH 7.3) for 10 minutes. The cells were then washed in PBS 3 times for 10 minutes each. Then the coverslips were placed in blocking solution containing 1% serum, 1% DMSO, and 1% Triton X-100 in 1 mL PBS for 30 minutes. Between each step the slides were washed 2 times for 10 minutes each in PBS. Next the coverslips were placed in primary antibodies in blocking solution overnight. The primary antibodies included mouse anti ED-1 (Serotec; 1:100), mouse anti OX-42 (Cederlane; 1:100), rabbit anti Iba-

1 (WAKO; 2 ug/mL), rabbit anti PGP 9.5 (Ultraclone; 1:100), and mouse anti GFAP (Sigma; 1:100). Lastly, the secondary antibody in blocking solution was placed on the coverslips for 4 hours. The secondary antibodies included donkey anti rabbit Texas Red (1:100), goat anti mouse FITC (1:100), and donkey anti mouse Texas Red (1:100). The coverslips were then inverted onto glass slides with Vectashield (Vectorlabs) for visualization under a fluorescence microscope.

3.4 Labelling for Allograft and Tail Vein Injection

When HAPI or rMC-1 cells reached 70% confluency, serum free media was added. The cells were grown until they were 90% confluent and were labelled with wheat germ agglutinin conjugated Texas Red fluorescence (WGA-TR; W831; Molecular Probes, Eugene, OR) by washing the cells in WGA-TR diluted in PBS for 10 minutes. The WGA was then removed and the cells were washed 3 times with PBS. Then the cells were prepared for injection, imaging, or cell counting.

3.5 LPS Activation of HAPI Cells

The cells were grown (as described above, except serum free media was added at least 24 hours before experiments were to take place) until they were 70% confluent and then were hyper-activated by adding 1µg/mL LPS (Escherichia coli 055:B5; L2637; Sigma Aldrich; St. Louis, MO) in fresh media for 24 hours. HAPI cells were washed 5 times in PBS before being labelled with WGA-TR (W831; Molecular Probes, Eugene, OR) in PBS for 10 minutes. The WGA was then removed and the cells were washed 3 times with PBS.

3.6 LAL Endotoxin Assay to Measure LPS levels

The amount of LPS in the HAPI cell solution for injection was measured by using the Limulus Amebocyte Lysate (LAL) assay (GeneScript; Piscataway, USA). Briefly explained, the endotoxin standard, HAPI cell solution, and LPS activated HAPI cell solution was mixed with the LAL solution and incubated for 45 minutes in a 37^{o}C water bath. Then the stop solution and colour stabilizers were added to bring about a colour gradient. The absorbance of the colour gradient was measured at 545 nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific; Wilmington, Delaware).

3.7 Minocycline Treatment of HAPI Cells

The cells were grown (as described above, except serum free media was added at least 24 hours before experiments were to take place) until they were 70% confluent and then were treated with 10 µg/mL minocycline (Sigma Aldrich; St. Louis, MO) in fresh media for 1 hour. HAPI cells were washed 5 times in PBS before being labelled with WGA-TR in PBS for 10 minutes. The WGA was then removed and the cells were washed 3 times with PBS. Then the cells were prepared for injection, imaging, or cell counting.

3.8 Animals

Adult, female Sprague–Dawley rats (225–250 g; Charles River, Wilmington, MA) that were free of common pathogens were used in all experiments. Animals were cared for according to the guidelines of the Canadian Council on Animal Care. The animals were kept on a 12 hour light cycle and had

access to food and water *ad libitum.* In all experiments the rats were anesthetized with an intraperineal injection of 7% chloral hydrate (Thermo Fisher Scientific, Ottawa, ON; 0.42 g/kg of body weight) during experimental procedures. An ophthalmic eye lubricant (Lacri-Lube; Allergan, Markham, ON, Canada) was applied to the eyes before surgery. Animals were kept warm on a heating blanket (38^{o}C). Animals were given subcutaneous injections of Anafen (Merial Canada, Baie D'Urfé, QC; 5 mg/kg) to minimize discomfort following surgery. They were also given 5 mL of saline subcutaneously and allowed to recover on a heating blanket.

3.9 Retrograde Labeling of Retinal Ganglion Cells

Retinal ganglion cells were retrogradely labeled before the optic nerve crush by injecting Fluorogold (FG; Fluorochrome LLC, Denver, CO) bilaterally into the superior colliculus as described by Koeberle and Ball (1998). The rat was placed on a stereotaxic frame (David Kopf M900, Tujunga, CA) and an incision was made on top of the head approximately 1-2 mm from the eyes and 1-2 mm before the ears. Once the incision was made, the transverse suture was located. A 1 mm hole was drilled bilaterally 2.5 mm rostral to lamda and 1.2 mm lateral to the sagittal sutures. Four microliters of FG was injected 3 mm deep into the parenchyma of the brain using a 10 μl Hamilton syringe (Hamilton M701, Reno, NV) with the aid of a microinjector (WPI UltraMicroPump, Sarasota, FL).

3.10 Intraocular Injections and Tail Vein Injections of Untreated HAPI cells

The HAPI cells were trypsinzed and centrifuged at 320 RPM for 5 minutes. Once there was a visible pellet of cells on the bottom of the vial, the cells were resuspended in PBS. Immediately before ONC, animals received HAPI cell injection into the vitreous chamber of the eye by a posterior route or a tail vein injection by the lateral vein route.

The sclera was punctured within 3 mm of the optic nerve with a 30 gauge needle. Then, 5 μL of the HAPI or rMC-1 cells solution (approximately 30,000 cells) was injected into the vitreous cavity with a 33 gauge Flexifil beveled needle (NF33FBV; World Precision Instruments, Sarasota, FL) on a WPI Nanofil Syringe (300329; World Precision Instruments, Sarasota, FL). Care was taken not to damage the lens or the anterior structures of the eye, which are known to secrete growth factors (Leon, Yin, Nguyen, Irwin, & Benowitz, 2000). Following intraocular injections, the needle was left in place for 2 minutes and withdrawn slowly. In the tail vein condition, a 25 gauge needle was used to injection 1 mL of HAPI or rMC-1 cell solution (approximately 5 million cells) into the lateral tail vein.

3.11 Intraocular Injections and Tail Vein Injections of Treated HAPI Cells

The HAPI cells grafting solution was created as described for injection of untreated HAPI cells. The sclera was punctured within 3 mm of the optic nerve with a 30 gauge needle. Then 5 μL of the HAPI cells solution (approximately 30,000 cells) was injected into the vitreous cavity with a fine, pulled micropipette

connected to a pico pump (WPI, Sarasota, FL.). Care was taken not to damage the lens or the anterior structures of the eye, which are known to secrete growth factors (Leon et al., 2000). Following intraocular injections, the needle was left in place for 1 minutes and withdrawn slowly. In the tail vein condition, a 25 gauge needle was used to injection 1 mL of HAPI cell solution (approximately 5 million cells) into the lateral tail vein.

3.12 Optic Nerve Injury

Optic nerve crush (ONC) or optic nerve transection (ONT) injury was done one week after FG injection and immediately after HAPI cells injection. The animals were prepared for surgery as previously described. An incision was made in the skin around the rim of the orbital bone to access the optic nerve. The orbital contents were retracted away and the rectus muscle was moved laterally. The eye was rotated temporally to expose the optic nerve. The optic nerve was either crushed by compressing the intradural optic nerve for 3 seconds (described in Kalesnykas et al., 2012) using Dumont SS fine forceps (Fine Science Tools 11203-23; North Vancouver, BC), or transected by removing the optic nerve from the dural sheath and cutting the nerve (described in Koeberle & Ball, 1998).

3.13 Tissue Preparation

The rats were euthanized between 2 and 28 days after injury or injection (2-28 days for BM88 experiment; 4, 7, and 14 days for all experiments using HAPI cells) by injecting a lethal dose (5 mL) of 7% chloral hydrate. The eyes were enucleated and the cornea and lens were dissected away. The eye cup was

fixed in 4% paraformaldehyde, 2% sucrose in Sorensen's phosphate buffer (0.1M, pH 7.3) for 2 hours. The eye cups and optic nerve were rinsed in sodium phosphate buffered saline (PBS; 0.1 M, pH 7.3, 0.9% NaCl) 3 times for 30 minutes each. The eyecups were placed in 30% sucrose in PBS at 4°C overnight. The eyecups were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrence, CA) and sectioned in a cryostat microtome (Leica Microsystems CM1900, Concord, ON) at -20°C. Transverse sections of 12 μm thickness were made for and placed on Superfrost Plus slides (Thermo Fisher Scientific, Ottawa, ON).

3.14 Labeling of Retinal Ganglion Cells with Brn3a Antibody

The retinal sections were washed in PBS 3 times for 5 minutes each. The retinas were incubated in a blocking solution overnight at room temperature. The blocking solution contained PBS with 10% normal donkey serum, 3% bovine serum albumin, 1% triton X-100, and 1% DMSO. Retinal ganglion cells were labelled with a Brn3a antibody, which is s known to label over 90% of RGCs (Nadal-Nicolás et al., 2009). The retinal sections were incubated in the Brn3a antibody (α goat; 1:200; Santa Cruz SC-31984 (C-20); immunizing protein human class IV POU domain protein; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in blocking solution over night at room temperature. To detect the primary antibody, donkey α goat Alexa 488 (1:200; Molecular Probes A-11055, Life Technologies, Burlington, ON) in blocking solution was placed on the retinal sections for 4 hours at room temperature. The sections were washed in PBS 3 times for 5

minutes each after each incubation period. The slides were coverslipped in Vectashield (H1000; Vector Labs, Burlington, ON) for visualization under an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON).

3.15 Labelling of Retinal Ganglion Cells with BM88 Antibody

Retinal sections were washed in PBS 3 times for 10 minutes each. Then blocking solution containing 1% normal donkey serum (Jackson ImmunoResearh Laboratories, West Grove, PA), 1% DMSO (BDH Chemicals, Toronto, ON), and 1% Triton X-100 (BDH Chemicals, Toronto, ON) in 1 mL PBS was put on the slides for 1 hour. Between each step the slides were washed 2 times for 10 minutes each in PBS. Next, rabbit α BM88 (sc-138749 (S11); Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:100) in blocking solution was put on the slides overnight. The primary antibody was detected with donkey α rabbit Texas Red (711-075-152; Jackson ImmunoResearh Laboratories, West Grove, PA; 1:100) in blocking solution was placed on the slides for 4 hours. The slides were coverslipped in Vectashield (H1000; Vector Labs, Burlington, ON) for visualization under an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON).

3.16 Co-localization of BM88 with Brn3a

The retinal sections were washed in PBS 3 times for 5 minutes each. The retinas were incubated in a blocking solution overnight at room temperature. The blocking solution contained PBS with 10% normal donkey serum, 3% bovine serum albumin, 1% triton X-100, and 1% DMSO. Retinal ganglion cells were

labelled with the Brn3a antibody. The retinal sections were incubated in the Brn3a antibody (α goat; 1:200; Santa Cruz SC-31984 (C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and BM88 (α rabbit; sc-138749 (S11); Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:100) in blocking solution over night at room temperature. The primary antibodies were detected using donkey α goat Alexa 488 (1:200; Molecular Probes A-11055, Life Technologies, Burlington, ON) and donkey α rabbit Alexa 568 (1:200; Molecular Probes A-10042, Life Technologies, Burlington, ON) in 1% normal donkey serum, 1% triton X-100, and 1% DMSO placed on the retinal sections for 4 hours at room temperature. The sections were washed in PBS 3 times for 5 minutes each after each incubation period. The slides were covered with a glass coverslip with Vectashield (H1000; Vector Labs, Burlington, ON) for visualization under an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON).

3.17 Measurement and Statistical Analysis of BM88 immunoreactive RGCs

Images were captured using an AxioCamMRm camera and AxioVision 4 software (Carl Zeiss Canada, Toronto, ON) and the number surviving RGCs were counted manually. The cells were counted and the staining intensities were measured from samples located in the mid-periphery of the retina between 50 and 150 μm from the optic nerve head. The staining intensity of the antibody labelling was measured using standardized camera settings (Zeiss 100W HBO9 at 20%; gamma 1.0; 31 msec exposure). Sections stained at the same time using the same reagents were used for intensity measurements. The exposure time of 31 msec

was established by using the AxioVision over/under exposure function so that all the pixels were within the minimum (28 days)/maximum (0 days) linear numerical values (0-255) in all samples measured. ImageJ software (National Institutes of Health, version 1.42j) was used to measure the average mean grey value of the whole cell on 8 bit images. Statistical analysis was done using GraphPad Prism (La Jolla, CA). Significance was determined by ANOVA analysis (alpha = 0.05) followed by Tukey's post-hoc test. All ± values were reported as 95% confidence intervals.

3.18 Calculating Area of Inflammation of Treated Eye Cups and Statistical Analysis

The area of inflammation was calculated using ImageJ (National Institutes of Health, version 1.42j). Before the eyes were sectioned, images of eye cups with intact retina were taking using a camera attached to a dissecting microscope. The scale in ImageJ was determined for each image and a boundary around the inflammation site was created manually. This was determined as the portion of the retina in the eye cup that was visibly swollen. The area of the retina within the boundary was calculated. Statistical analysis was done using GraphPad Prism (La Jolla, CA). Significance was determined by ANOVA analysis (alpha = 0.05) followed by Tukey's post-hoc test. All ± values were reported as 95% confidence intervals.

CHAPTER 4 – 8: RESULTS AND DISCUSSION

CHAPTER 4: RETINAL GANGLION CELL DEATH AND CELL MIGRATION AFTER ALLOGRAFT OF MICROGLIAL CELLS AND OPTIC NERVE INJURY

4.1 Results

The function of resting retinal microglia and over activated microglia in retinal neurodegeneration is not well understood. It has been argued whether or not activation of microglial cells after injury serves to be neuroprotective or neurotoxic. The nature and time-course of the injury may determine if retinal microglial cells acquire a neuroprotective or pro-inflammatory phenotype. HAPI (highly aggressive proliferating immortalized) cells are a immortalized microglial cell line, whose phenotype can be manipulated *in vitro*. The purpose of this study was to determine if cultured HAPI cells injected into the eye, or systemically, would migrate to the retina and optic nerve after optic nerve injury and how this would affect the survival of RGCs. It was my hypothesis that cells in a resting state would not result in any change in RGC numbers but that HAPI cells activated by the injury would be detrimental to the survival of RGCs. An overview of this chapter is in Appendix I.

4.1.1 Characterization of HAPI cells

HAPI (highly aggressive proliferating immortalized) cells are a spontaneously occurring, immortalized cell line that is derived from primary neonatal rat microglial cultures (Cheepsunthorn, Radov, Menzies, Reid, & Connor, 2001). HAPI cells were immunoreactive for microglial markers OX-42 (figure 4.1b) and Iba-1 (figure 4.1a), while not being reactive for the neuronal marker PGP 9.5 (figure 4.1d) and the astrocytic marker GFAP (figure 4.1c). The rMC-1 cells were immunoreactive for GFAP (figure 4.2c) but not for OX-42

(figure 4.2b), Iba-1 (figure 4.2a), and PGP 9.5 (figure 4.2d). Both rMC-1 and HAPI cells were negative for non-selective binding of only secondary antibodies.

4.1.2 Labelling HAPI Cells with Wheat Germ Aggulutinin

We needed to be able to track the HAPI cells and differentiate them from resident microglia. HAPI cells and rMC-1 cells were labelled with wheat germ agglutinin (WGA-OG or WGA-TR, respectively) and continued to fluoresce for 25 days after labelling with WGA. When HAPI cells were added to rMC-1 cells growing on a coverslip (previously labelled with a different colour of WGA), within one day, double labelled cells were visible. HAPI cells were most likely the double labelled cells because they were the only phagocytic cells in culture. However, to rule out the possibility that the WGA from one cell type was being leeched and then labelling the other cell type in the media, the cells were grown separately and 400 μL of media from one WGA type was added to the other. For example, rMC-1 cells labelled with WGA-TR and 400 μL of media from rMC-1 cells labelled with WGA-OG added to it. If the WGA was leeched into the media, then the cells should be labelled with both Texas Red and Oregon Green. However, the opposite was true and there was no leeching because the cells were labelled with only one type of WGA.

4.1.3 Migration of HAPI Cells

At 4, 7, and 14 days after HAPI cell injection, cells were only visible in the retina after intravitreal injection accompanied by an optic nerve crush (figure 4.4 and 4.8). HAPI cells were present in the optic nerve at 4, 7, and 14 days after

HAPI cell were injected into the tail vein and the optic nerve was crushed (figure 4.6 and 4.10). In the non-injured condition, regardless of the method of injection, there were no HAPI cells seen in the retina or optic nerve (figure 4.3, 4.5, 4.7, and 4.9).

4.1.4 RGC Loss after Optic Nerve Crush

The optic nerve crush (ONC) is an injury model that results in the gradual loss of RGCs over time (figure 4.11). Non-injured retinas had 54.75 ± 1.87 RGCs/mm (n=6; figure 4.11D and 4.12). Four days after ONC there were 44.90 ± 3.81 RGCs/mm (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$), an expected loss of 18.0 % of RGCs. At 7 days after ONC there was an expected loss of 37.7 % of RGCs with 34.12 ± 1.79 RGCs/mm left (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$; figure 4.11B and 4.12). After 14 days of ONC there was a loss of 65.4 % of RGCs with 18.9 ± 1.05 RGCs/mm remaining (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$; figure 4.11C and 4.12). These values were used as controls for experiments introducing HAPI cells into the vitreous or tail vein.

4.1.5 No RGC loss after rMC-1 Intravitreal or Tail Vein Injection

There was no significant loss of RGCs after intravitreal or tail vein injection of rMC-1 cells over the 14 day period (ANOVA/Tukey's post-hoc test; $p > 0.05$; figure 4.13 and 4.14). Control retinas had 58.40 ± 4.71 RGCs/mm (n=6). Four days after rMC-1 injection into the tail vein there were 54.25 ± 7.94 RGCs/mm (n=2). There were 64.39 ± 11.64 RGCs/mm (n=2) and 61.45 ± 6.25 RGCs/mm 7 and 14 days (respectively) after rMC-1 injections into the tail vein.

Similarly, there were 66.72 ± 8.25 RGCs/mm (n=2) 4 days after intravitreal injection of rMC-1 cells and 61.92 ± 6.55 RGCs/mm (n=2) 7 days after injection. Fourteen days after intravitreal injection there were 65.41 RGCs/mm (n=2) left.

4.1.6 RGC Loss after Intravitreal Injection of HAPI Cell

Four days after HAPI cells were injected into the vitreous without any injury to the optic nerve, the RGC density was 56.22 ± 2.70 RGCs/mm (n=6; figure 4.12). This was not significantly different from the control (ANOVA/Tukey's post-hoc test; $p > 0.05$). Similarly, at 7 days, the RGC density was 54.54 ± 2.15 RGCs/mm (n=6; figure 4.12). This also was not significantly different from the control (ANOVA/Tukey's post-hoc test; $p > 0.05$). However, at 14 day after HAPI cell injection into the vitreous there was an RGC density of 45.86 ± 1.52 RGCs/mm (n=6; figure 4.12). This was significantly different than control retinas (ANOVA/Tukey's post-hoc test, $p \leq 0.001$; figure 4.12). In addition, when rMC-1 cells were injected into the vitreous or tail vein, there was no significant loss of RGCs over the 14 day period.

Four days after HAPI cells were injected into the vitreous and ONC injury, there was a RGC density of 37.45 ± 2.83 RGCs/mm (n=6; figure 4.12). This resulted in 16.6 % more loss of RGCs 4 days after intravitreal injections of HAPI cells and ONC than the loss that would be expected from ONC alone (ANOVA/Tukey's post-hoc test, $p \leq 0.01$; figure 4.12). Seven days after intravitreal injection of HAPI cells and ONC, there was greater loss of RCGs. These retinas had a RGC density of 20.39 ± 2.44 RGCs/mm (n=6; figure 4.12).

This is a loss of 40.24 % more RGCs 7 days after intravitreal injection of HAPI cells and ONC than would be expected after ONC alone (ANOVA/Tukey's post-hoc test, $p \leq 0.001$; figure 4.12). This effect was lost after 14 days of injury. RGC density of retinas 14 days after intravitreal injection of HAPI cells and ONC was 18.26 ± 3.31 RGCs/mm (n=6; figure 4.12). This was not significantly different than the RGC densities for retinas 14 days after ONC (ANOVA/Tukey's post-hoc test; $p > 0.05$).

It is interesting to note that the RGC density for the retinas 4 days after intravitreal injection of HAPI cells and accompanied by an ONC was not statistically different than the densities for retinas 7 days after ONC alone (ANOVA/Tukey's post-hoc test; $p > 0.05$; figure 4.12). Also, the RGC densities for the retinas 7 days (ANOVA/Tukey's post-hoc test; $p > 0.05$) and 14 days (ANOVA/Tukey's post-hoc test; $p > 0.05$) after intravitreal injection of HAPI cells and accompanied by an ONC was not statistically different than the densities for the retinas 14 days after ONC alone.

4.1.7 RGC Loss after Tail Vein HAPI Cell Injection

Four days after injection of HAPI cells into the tail vein without ONC resulted in a RGC density of 54.29 ± 3.25 RGCs/mm (n=6; figure 4.15). This was not statistically different from the control (ANOVA/Tukey's post-hoc test; $p > 0.05$). The RGC density 7 days after tail vein injection of HAPI cells without injury was 55.31 ± 2.10 RGCs/mm (n=6; figure 4.15). This was not statistically different from the control (ANOVA/Tukey's post-hoc test; $p > 0.05$). However,

14 days after the injection of HAPI cells in the tail vein the RGC density was 47.95 ± 2.02 RGCs/mm (n=6; figure 4.15). Similar to when HAPI cells were injected into the vitreous for 14 days, there was a statistically significant loss of RGCs (ANOVA/Tukey's post-hoc test, $p \leq 0.001$) 14 days after HAPI cells were injected into the tail vein in the absence of an injury.

The density of surviving RGCs 4 days after the injection of HAPI cells in the tail vein and accompanied by an ONC was 44.65 ± 2.62 RGCs/mm (n=6; figure 4.15). This was not statistically different from the RGC density 4 days after ONC (ANOVA/Tukey's post-hoc test; $p > 0.05$). However, the RGC density 7 days after tail vein injection and ONC was 27.36 ± 1.80 RGCs/mm (n=6; figure 4.15). This represented a significant loss of 19.81 % more RGCs than are lost 7 days after ONC alone (ANOVA/Tukey's post-hoc test, $p \leq 0.001$). The RGC density 14 days after tail vein injection and ONC was 14.13 ± 1.58 RGCs/mm (n=6; figure 4.15). This represented a significant loss of 25.40 % more RGCs than that would be lost after ONC alone (ANOVA/Tukey's post-hoc test, $p \leq 0.01$). Tail vein injections of HAPI cells accompanied by an ONC resulted in a greater loss of RGCs compared to what would be expected after ONC alone over a 14 day period.

4.2 Discussion

4.2.1 Migration of HAPI Cells

The role of systemic immune systems in the CNS has been controversial because it is traditionally seen as an immune privileged location due to the

existence of a blood-brain-barrier (BBB). It is thought that the BBB is compromised after CNS injury and may allow blood-derived macrophages to infiltrate the CNS, contributing to the pathology (Lucin & Wyss-Coray, 2009). However, it is also argued that blood-derived macrophages are also found in injury models where the BBB is not affected, such as after lesion of the nerve innervating the hypoglossal nucleus (Yong & Rivest, 2009). Normally, it is difficult to distinguish blood-derived macrophages from resident microglia because they both have similar morphologies and phenotypes (Lucin & Wyss-Coray, 2009). However, by injecting exogenously labelled cells into the systemic system, we can observe their effect.

When HAPI cells were injected into the vitreal chamber, they only migrated to the retina if there was some injury signal for the cells to follow. If the cells were injected into a normal eye, there were no cells visible in the retina. HAPI cells were also not visible in the retina after tail vein injection. However, HAPI cells were visible at the optic nerve when injected into the tail vein, but only if there was an injury.

HAPI cells 4 days after injection in the tail vein and ONC were located at the crush site but were fewer in number than in the 7 day condition. At 7 days after HAPI cell injection in the tail vein accompanied by an ONC there were HAPI cells at the crush site and they spread out to the periphery along the edges. The most HAPI cells were seen at 7 days after injury. By 14 days, the HAPI cells were more scattered throughout the optic nerve. The pattern of HAPI cell

migration and number coincides with the observation that the greatest amount of death of RGCs was seen 7 days after tail vein injection accompanied by an ONC. The majority of the death of RGCs also happened 7 days following injected of the HAPI cells into the vitreous when there was an ONC. This suggests that HAPI cells are activated by day 4 but between day 4 and 7 arrive at the injury site and start to exert their effect.

4.2.2 Loss of RGCs after Injection of HAPI Cells

The only significant loss of RGCs when HAPI cells were injected into the vitreous or tail vein in the absence of injury was seen at the 14 day time point (figure 4.12 and 4.15). There were no HAPI cells physically located in the retina (figure 4.3C and 4.5C) or at the optic nerve (figure 4.7C and 4.9C) at 14 days without injury. However, this loss was not due to the injection of exogenous cells because there was no loss of RGCs when a different glial cell line (rMC-1) was injected into the tail vein or vitreous. This suggests that the injury may be due to secreted factors released from the microglial cells. Microglia produce many factors that are capable of inducing cell death, such as reactive oxygen species and pro-inflammatory cytokines (Lucin & Wyss-Coray, 2009). Inhibition of these factors may contribute to the survival of neurons, which is often seen with the beneficial effects of NSAIDs on neurodegenerative diseases (Lucin & Wyss-Coray, 2009).

The role of microglia after injury or neurodegeneration has been controversial. They can be neuroprotective and have reparative effects or be pro-

inflammatory and execute the death of neuronal cells (Lucin & Wyss-Coray, 2009; Yong & Rivest, 2009). Microglia play a role in killing dopaminergic cells in Parkinson's disease and may contribute to the neurodegeneration seen in mouse models of Amyotrophic lateral sclerosis (Boillée et al., 2006; Mount et al., 2007). When HAPI cells were injected into the vitreous of an eye and then the optic nerve was crushed there was a loss of 17% more RGCs at 4 days and 40% more RGCs at 7 days than that would be expected after ONC alone at those time points. At 14 days, there was no additional loss of RGCs. The majority of the death of RGCs was seen between 4 and 7 days. In addition, the density of RGCs at 4 days after intravitreal injection of HAPI cells and ONC was comparable to the density of cells at 7 days after ONC (figure 4.4, 4.10). The RGC density at 7 and 14 days after intravitreal injection of HAPI cells and ONC was comparable to the density of cells at 14 days (figure 4.4, 4.12). Initially, HAPI cells injected into the vitreous accelerated the death of RGCs but it leveled off after 14 days. This suggests that HAPI cells without any previous activation clean up dead and dying cells but do not kill any cells not destined to die after 14 days.

When HAPI cells were injected into the tail vein and there was an ONC injury, there was no additional loss of RGCs at 4 days. However, there was a loss of 20% more RGCs at 7 days and a loss of 25% more RGCs at 14 days than would be expected after ONC alone at those time points (figure 4.6 and 4.15). This suggests that it takes longer for the loss of RGCs to accelerate when the cells are injected into the systemic system. It takes 7 days for the additional loss of

RGCs to begin and continues to gradually accelerate over a 14 day period. The density of RGCs may level off at time points beyond 14 days similar to that seen in the intravitreal injections. The death of RGCs was less severe in the tail vein condition than the intravitreal condition. This may suggest that secreted factors and phagocytosis together mediate the role of RGC death by microglia after injury. This biphasic rate of RGC loss was also observed in other models, such as in laser-induced chronic ocular hypertension. It was noted that there is initially a rapid loss of RGCs of 12% per week for the first 3 weeks followed by a slower loss of 2% per week (WoldeMussie, Ruiz, Wijono, & Wheeler, 2001). It is possible that there is more local microglial involvement during the fast phase of loss and then more involvement of systemic macrophages during the slow phase of loss.

These experiments provide a novel method of investigation for the role of microglial activation in neurodegenerative disease. By using this novel technique of allografting HAPI cells, we can use different substances to activate the HAPI cells without causing toxicity to the animal or activating cells other than microglia. The exact role of microglia after injury is not well understood, particularly whether their activation is dualistic in nature. The following experiments in this thesis may help pinpoint the action of microglial cells after injury and help determine the conditions in which microglial cells are neurotoxic or neuroprotective.

4.3 Figures and Tables

Figure 4.1: Microglial, glial, and neuronal markers on cultured HAPI cells. Cultured HAPI cells immunolabelled with (A) a marker for microglial cells (rabbit anti Iba-1; WAKO; 2 ug/mL), (B) a marker for CD11 b/c macrophages and microglia (mouse anti OX-42; Cederlane; 1:100), (C) a marker of glial cells (mouse anti GFAP; Sigma; 1:100) (D) a neuronal marker (rabbit anti PGP 9.5; Ultraclone; 1:100), and (E) a secondary only control. There was positive staining of HAPI cells for microglia markers (a and b).

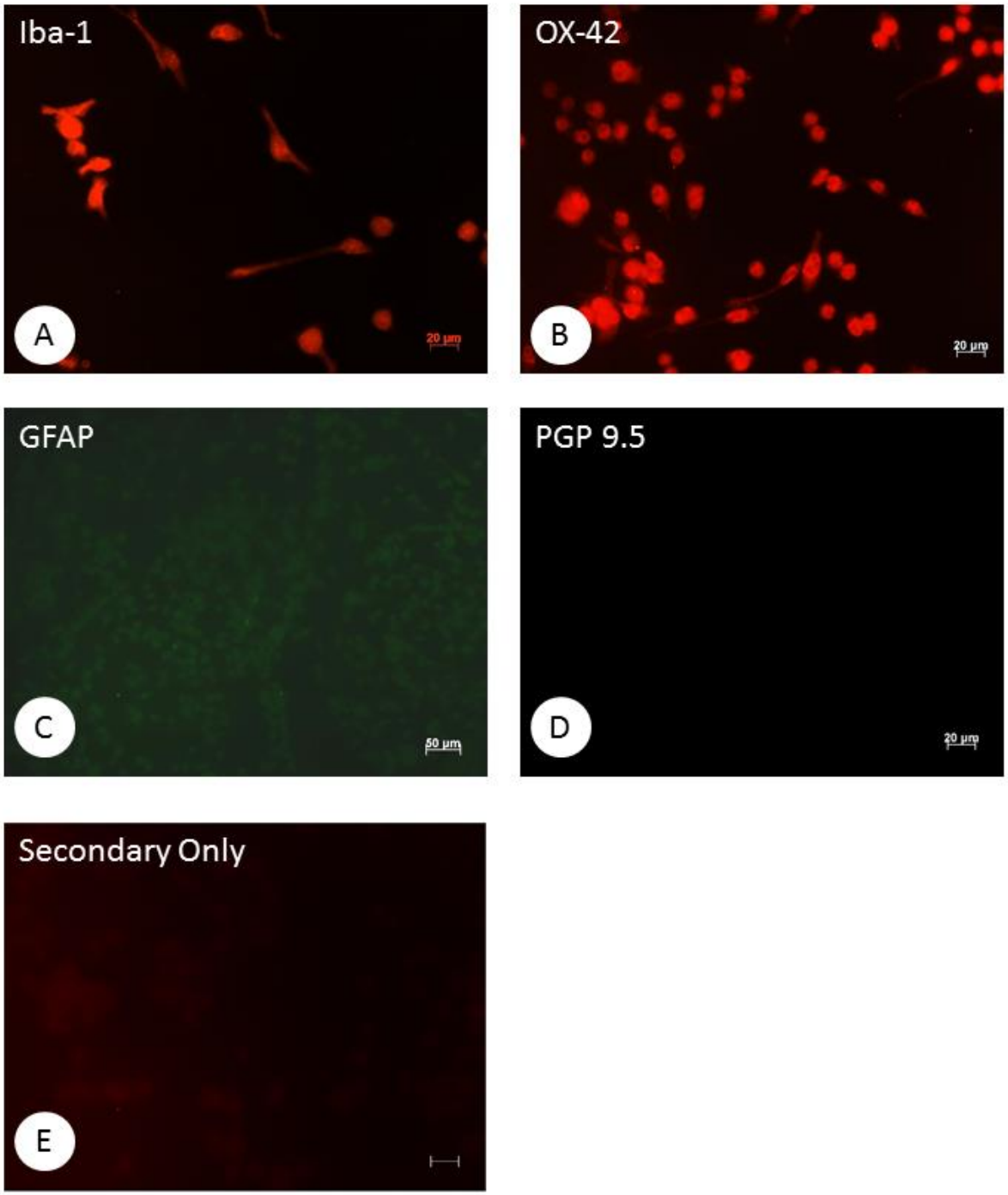

Iba-1
A
20 µm
OX-42
B
20 µm
GFAP
C
50 µm
PGP 9.5
D
20 µm
Secondary Only
E

Figure 4.2: Microglial, glial, and neuronal markers on cultured rMC-1. Cultured rMC-1 cells immunolabelled with (A) a marker for microglia cells (rabbit anti Iba-1; WAKO; 2 ug/mL), (B) a marker for CD11 b/c macrophages and microglia (mouse anti OX-42; Cederlane; 1:100), (C) a marker of glial cells (mouse anti GFAP; Sigma; 1:100) (D) a neuronal marker (rabbit anti PGP 9.5; Ultraclone; 1:100), and (E) a secondary only control. There was positive staining of rMC-1 cells for glial markers (C).

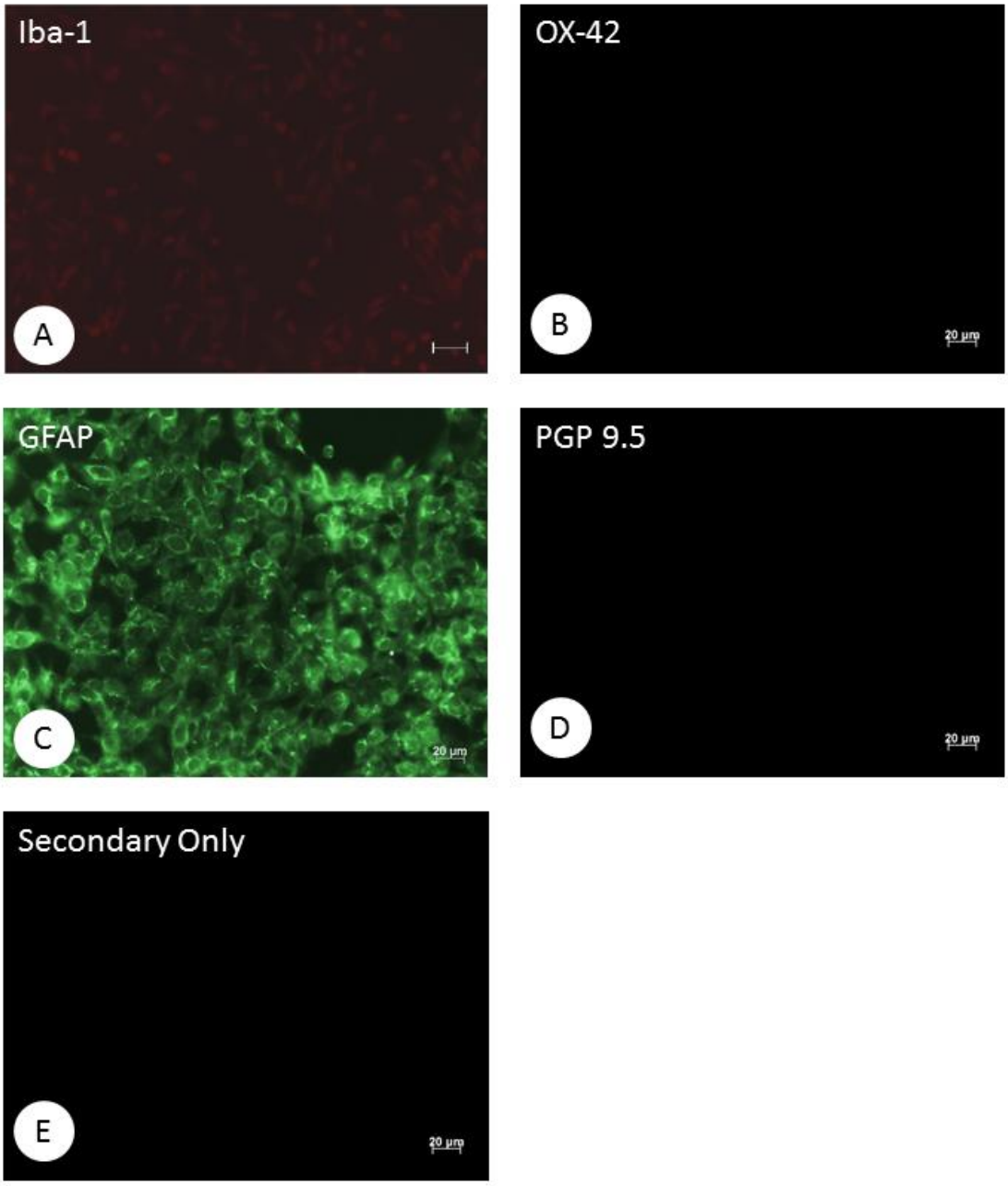

Iba-1
A
OX-42
B
20 µm
GFAP
C
20 µm
PGP 9.5
D
20 µm
Secondary Only
E
20 µm

Figure 4.3: 12 µm thick frozen transverse sections of retinas with fluorogold (labelling RGCs in gold) that had received intravitreal injections of HAPI cells and no optic nerve crush. (A) Retinas collected 4 days after HAPI cells were injected. **(B)** Retinas collected 7 days after HAPI cells were injected. **(C)** Retinas collected 14 days after HAPI cells were injected. **(D)** Control retinas where no HAPI cells were injected. Only at 14 days there was a statistically significant (ANOVA and Tukey's post hoc test) loss of retinal ganglion cells as compared to the control.

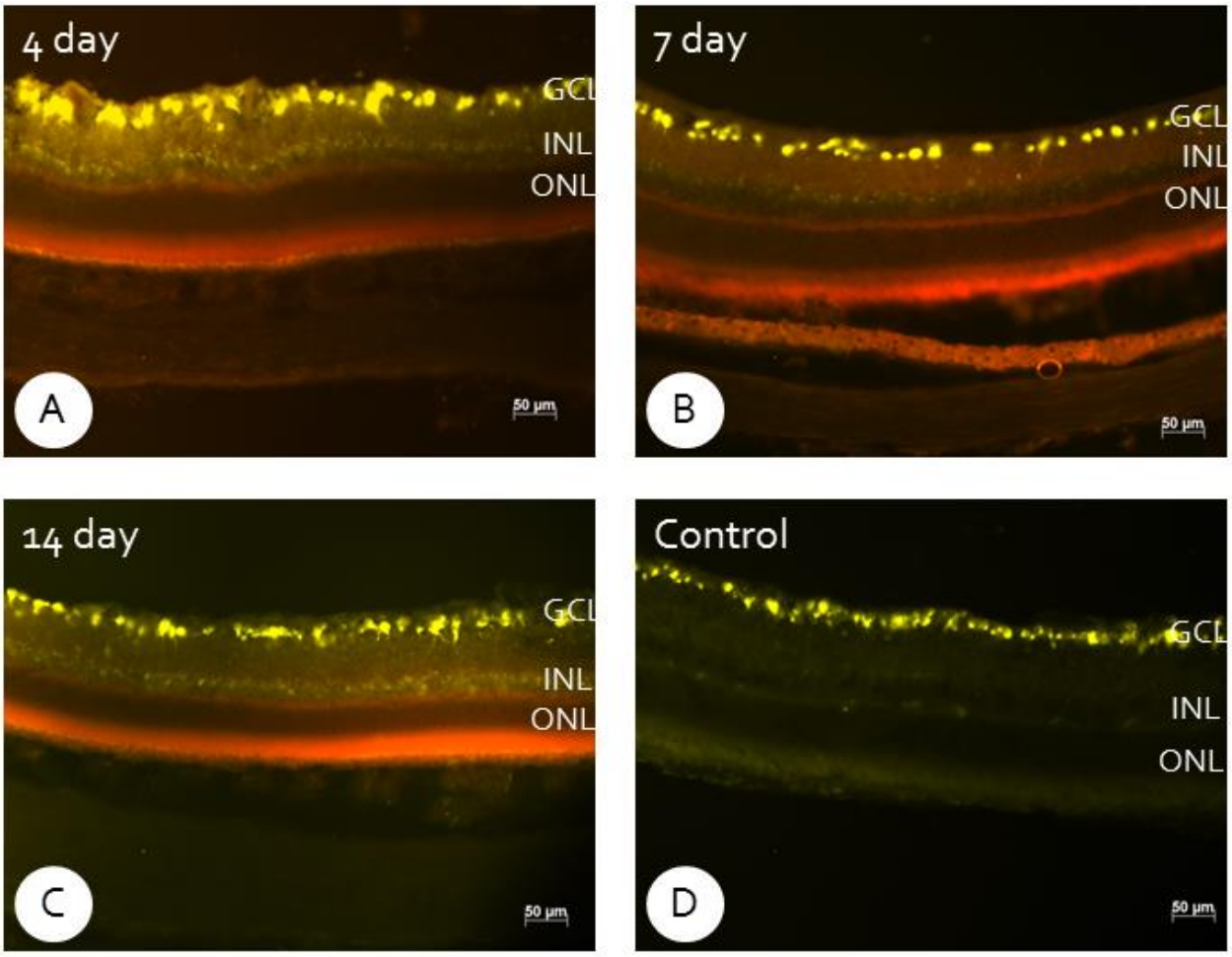

4 day
GCL
INL
ONL
A
50 µm
7 day
GCL
INL
ONL
B
50 µm
14 day
GCL
INL
ONL
C
50 µm
Control
GCL
INL
ONL
D
50 µm

Figure 4.4: 12 µm thick frozen transverse sections of retinas with fluorogold (labelling RGCs in gold) that had received intravitreal injection of HAPI cells (labelled with WGA-TR; red) followed by optic nerve crush. (A) 4 days after HAPI cell injection and optic nerve crush. HAPI cells were seen in the vitreous near the ganglion cell layer (white arrows). There was a loss of retinal ganglion cells comparable to (B). **(B)** Control retina 7 days after optic nerve crush. **(C)** 7 days after HAPI cells injection and optic nerve crush. More HAPI cells were seen in the vitreous near the ganglion cell layer (white arrows). There was a loss of retinal ganglion cells comparable to (E). **(D)** 14 days after HAPI cells injection and optic nerve crush. Fewer HAPI cells were seen in the retina (white arrows).There was a loss of retinal ganglion cells comparable to (E). **(E)** Control retina 14 days after optic nerve crush.

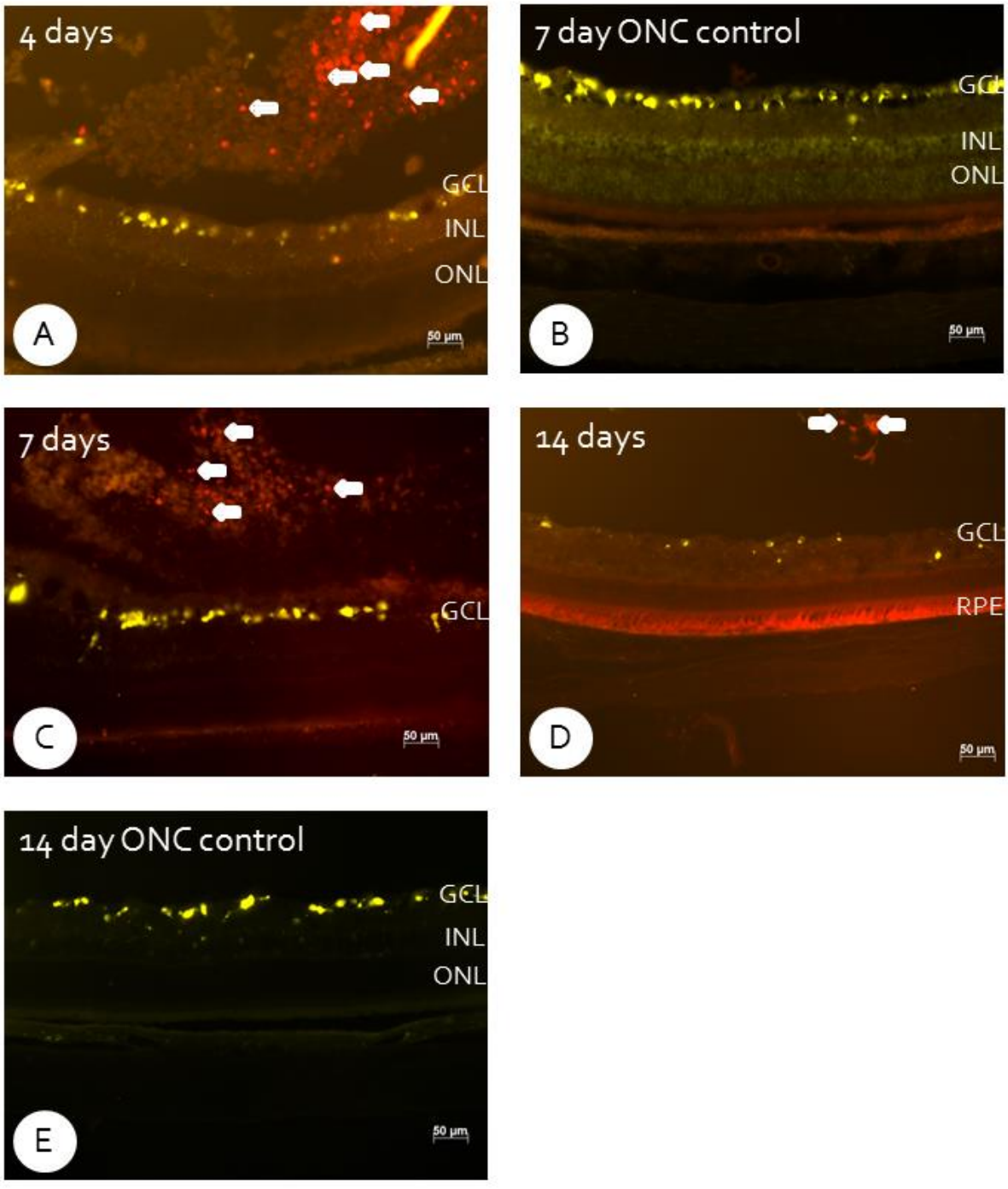

4 days
GCL
INL
ONL
A
50 µm
7 day ONC control
GCL
INL
ONL
B
50 µm
7 days
GCL
C
50 µm
14 days
GCL
RPE
D
50 µm
14 day ONC control
GCL
INL
ONL
E
50 µm

Figure 4.5: 12 µm thick frozen transverse sections of retinas with fluorogold (gold) that had received tail vein injections of HAPI cells and no optic nerve crush. (A) Retinas collected 4 days after HAPI cells were injected. **(B)** Retinas collected 7 days after HAPI cells were injected. **(C)** Retinas collected 14 days after HAPI cells were injected. **(D)** Control retinas where no HAPI cells were injected. Only at 14 days there was a statistically significant (ANOVA and Tukey's post hoc test) loss of retinal ganglion cells as compared to the control. There are no HAPI cells (labelled in WGA-TR; red) in the retinal sections after tail vein injections.

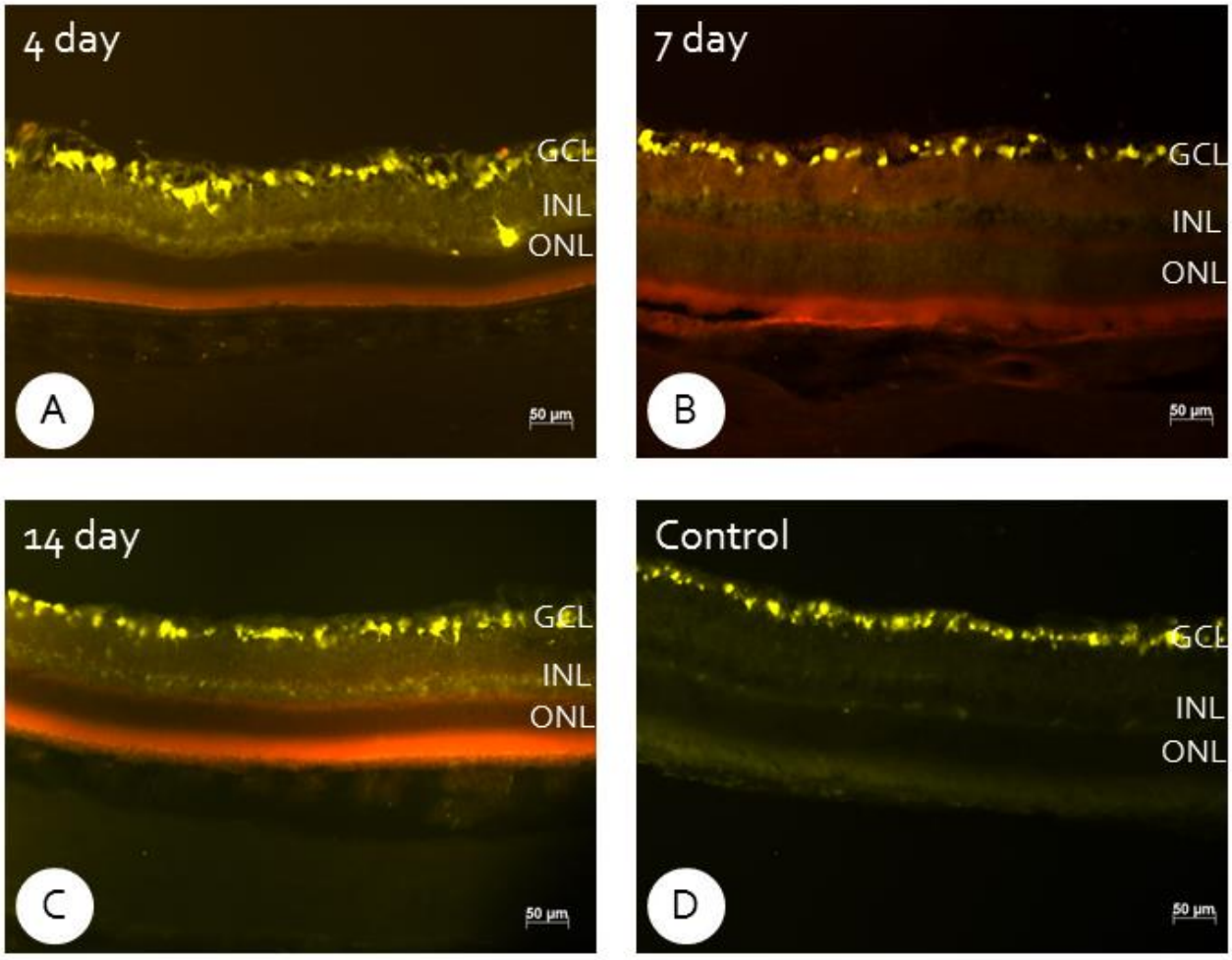

4 day
GCL
INL
ONL
A
50 µm
7 day
GCL
INL
ONL
B
50 µm
14 day
GCL
INL
ONL
C
50 µm
Control
GCL
INL
ONL
D
50 µm

Figure 4.6: 12 µm thick frozen transverse sections of retinas with fluorogold (labelling RGCs in gold) that had received tail vein injection of HAPI cells followed by optic nerve crush. (A) 4 days after HAPI cell injection and optic nerve crush. **(B)** Control retina 4 days after optic nerve crush. **(C)** 7 days after HAPI cells injection and optic nerve crush. There was a significant loss of RGCs as compared to its control (D). **(D)** Control retina 7 days after optic nerve crush. **(E)** 14 days after HAPI cells injection and optic nerve crush. **(F)** Control retina 14 days after optic nerve crush. There was no significant loss of RGCS at 4 days. There were also no HAPI cells present in the retina in any of the conditions.

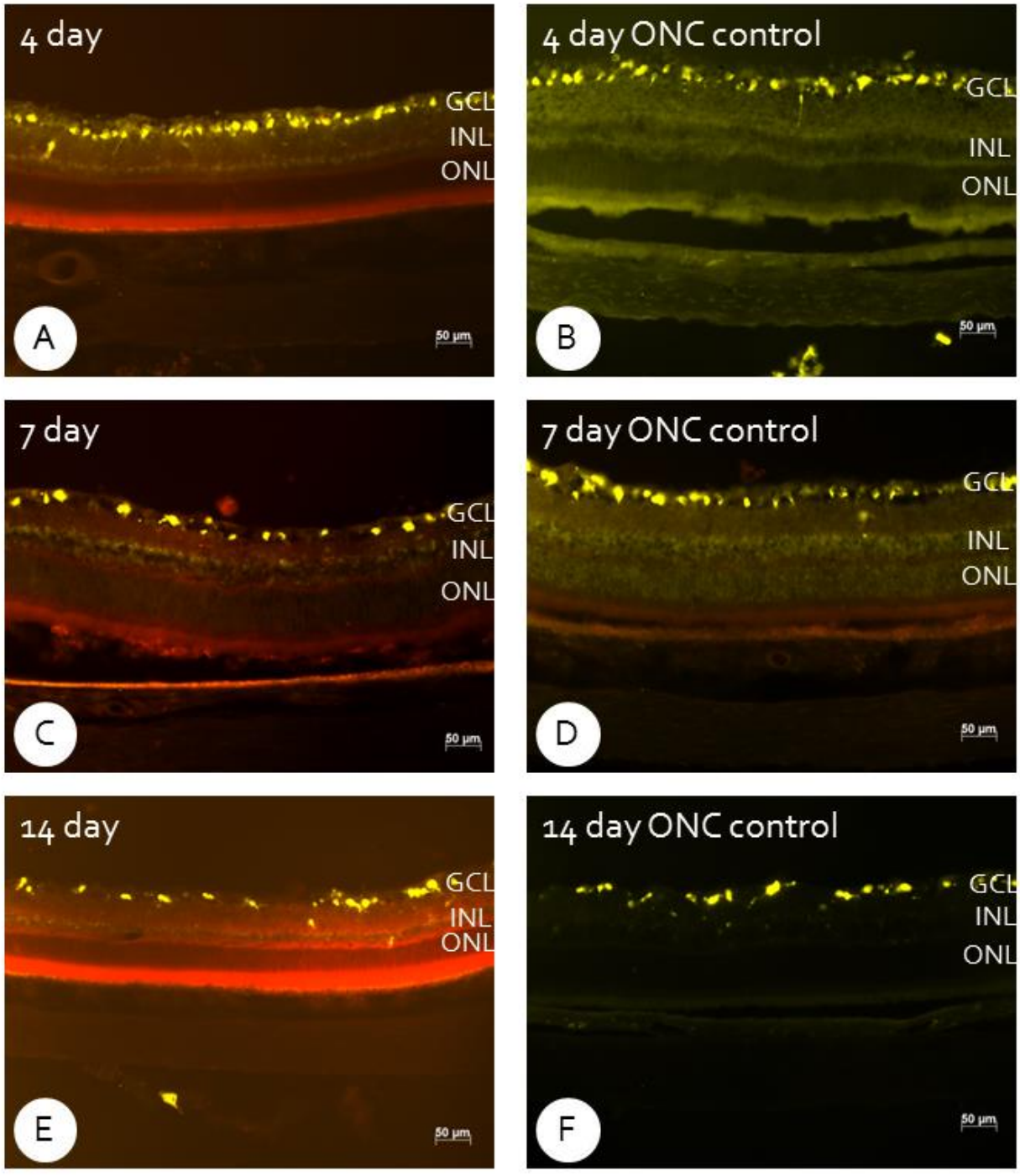

4 day
GCL
INL
ONL
A
50 µm
4 day ONC control
GCL
INL
ONL
B
50 µm
7 day
GCL
INL
ONL
C
50 µm
7 day ONC control
GCL
INL
ONL
D
50 µm
14 day
GCL
INL
ONL
E
50 µm
14 day ONC control
GCL
INL
ONL
F
50 µm

Figure 4.7: 12 µm thick frozen sections of optic nerve with fluorogold (gold) that had received intravitreal injection of HAPI cells (red) without optic nerve crush. (A) 4 days after HAPI cell injection. **(B)** 7 days after HAPI cells injection. **(C)** 14 days after HAPI cells injection. There were no HAPI cells present in the optic nerve after intravitreal injections.

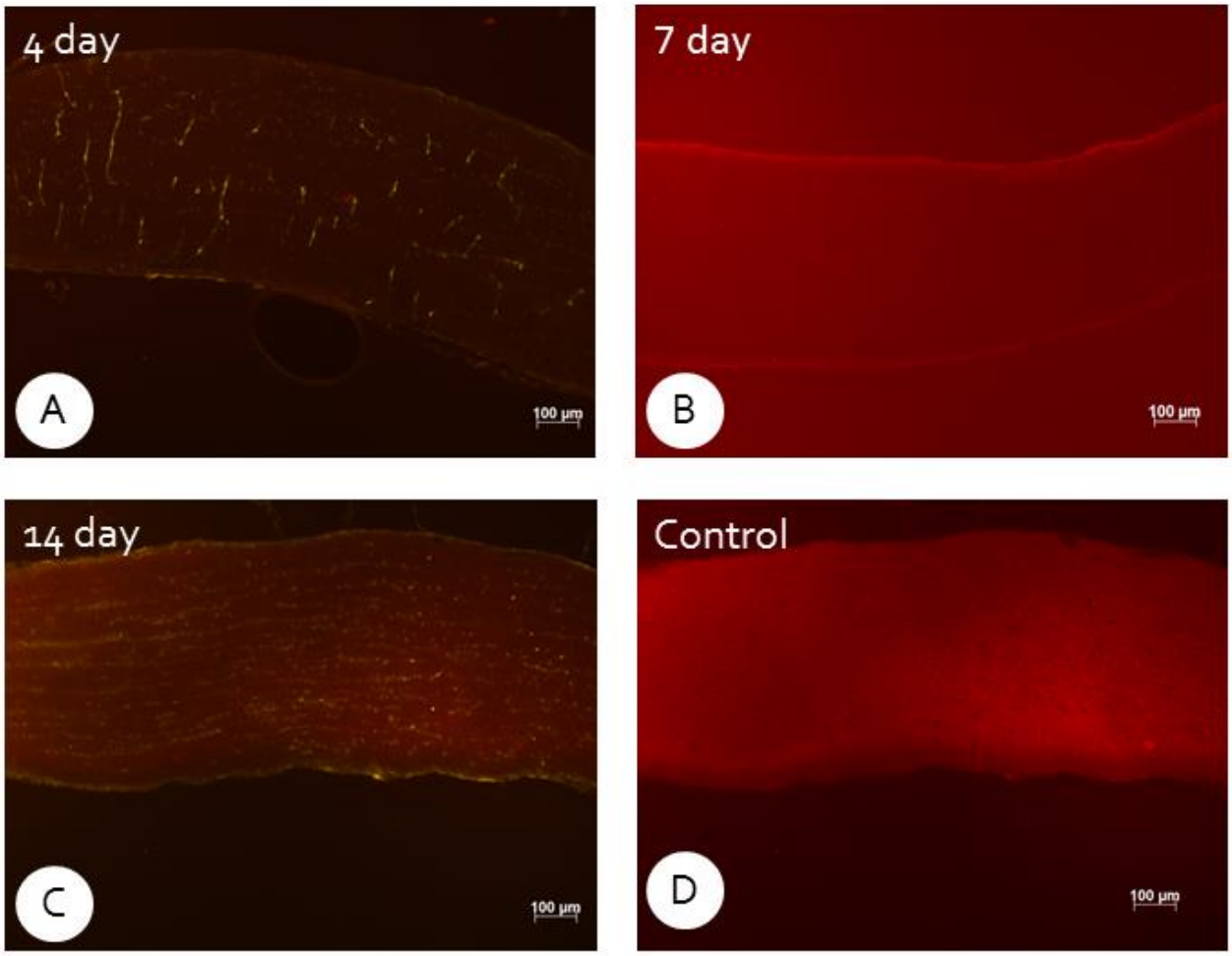

4 day
A
100 µm
7 day
B
100 µm
14 day
C
100 µm
Control
D
100 µm

Figure 4.8: 12 µm thick frozen sections of optic nerve with fluorogold (gold) that had received intravitreal injection of HAPI cells (red) followed by optic nerve crush (ONC). (A) 4 days after HAPI cell injection and ONC. **(B)** 7 days after HAPI cells injection and ONC. **(C)** 14 days after HAPI cells injection and ONC. There were no HAPI cells present in the optic nerve after intravitreal injections.

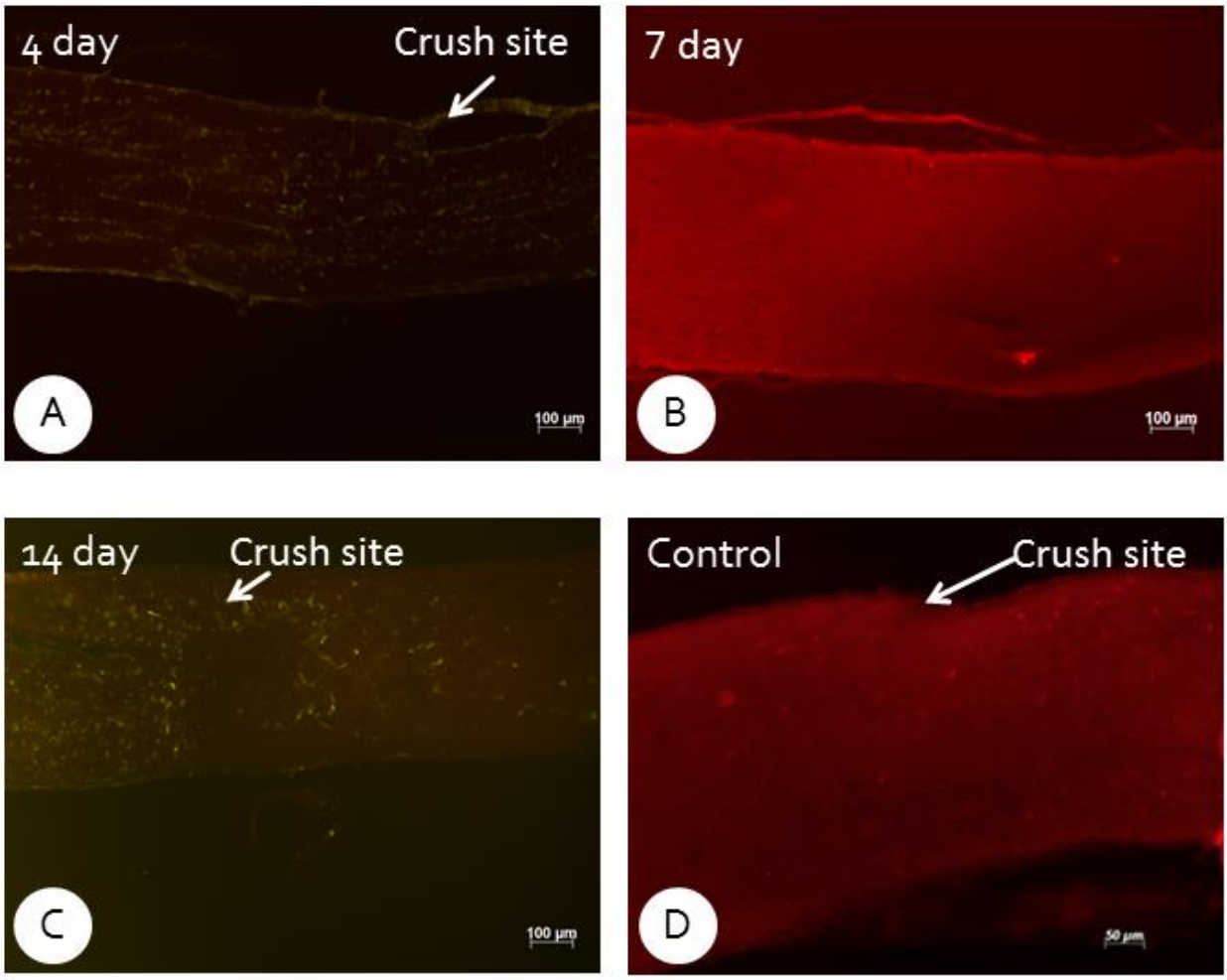

4 day
Crush site
A
100 µm
7 day
B
100 µm
14 day
Crush site
C
100 µm
Control
Crush site
D
50 µm

Figure 4.9: 12 µm thick frozen sections of optic nerve with fluorogold (gold) that had received tail vein injection of HAPI cells (red) without optic nerve crush. (A) 4 days after HAPI cell injection. **(B)** 7 days after HAPI cells injection. **(C)** 14 days after HAPI cells injection. There were no HAPI cells present in the optic nerve after tail vein injection in the absence of injury.

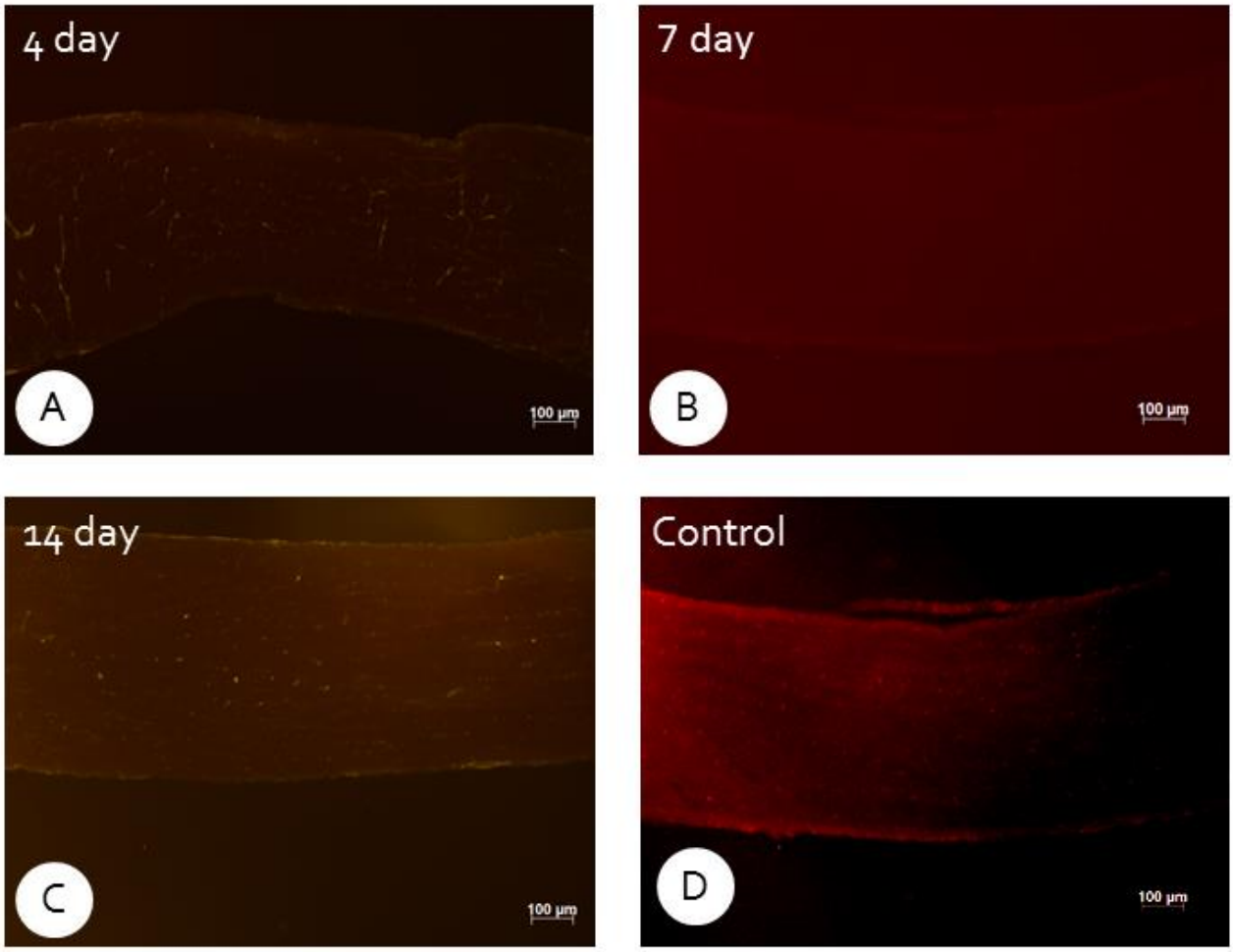

4 day
A
100 µm
7 day
B
100 µm
14 day
C
100 µm
Control
D
100 µm

Figure 4.10: 12 µm thick frozen transverse sections of optic nerves with fluorogold (gold) that had received tail vein injections of HAPI cells (red) and optic nerve crush. (A) Optic nerves collected 4 days after HAPI cells were injected. Few HAPI cells can be seen along the crush site. **(B)** Optic nerves collected 7 days after HAPI cells were injected. HAPI cells can be seen along the crush site and edges. **(C)** Optic nerves collected 14 days after HAPI cells were injected. Very few HAPI cells can be seen spread across the optic nerve. **(D)** Control optic nerves in rats where no HAPI cells were injected. No HAPI cells are present.

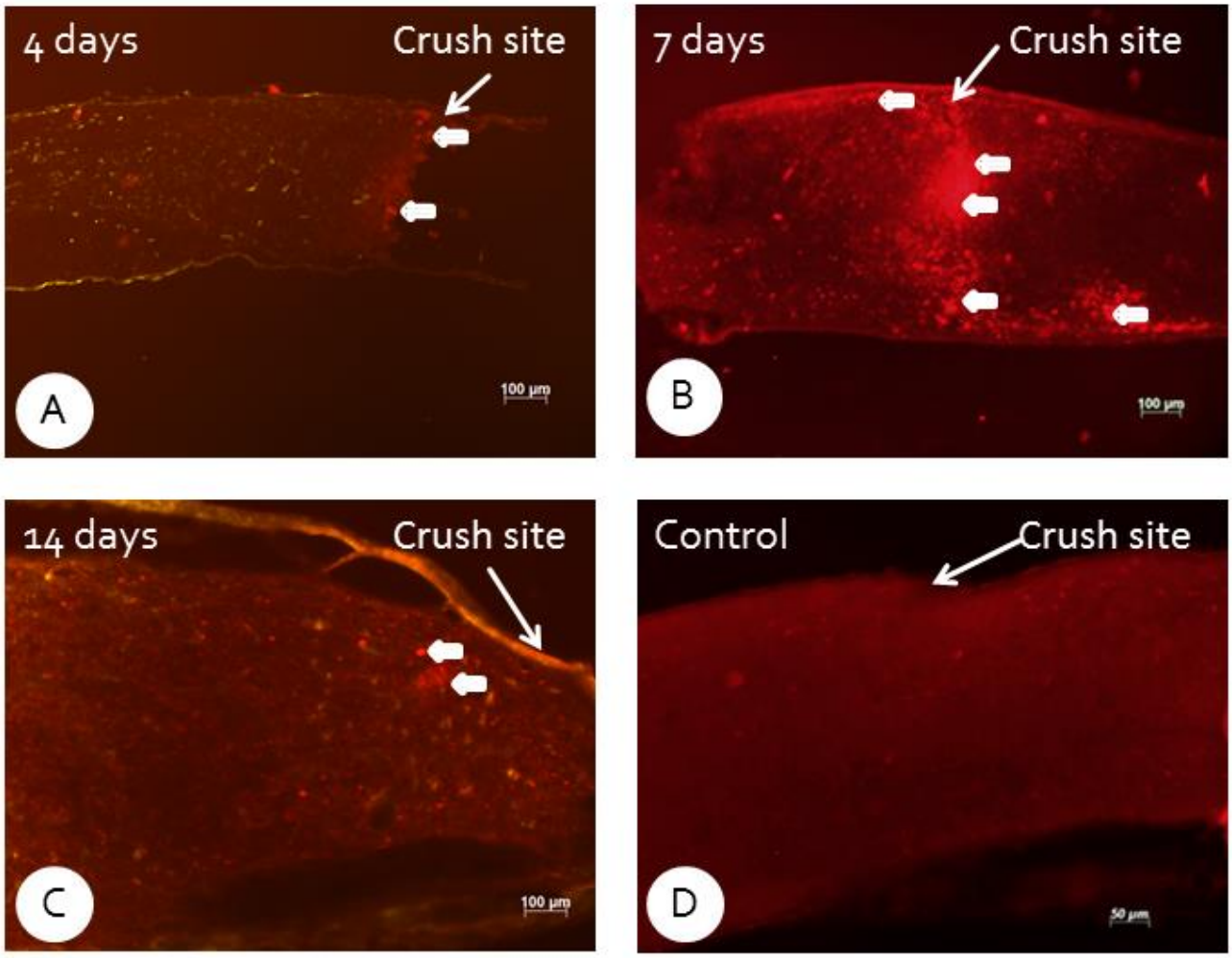

4 days
Crush site
A
100 µm
7 days
Crush site
B
100 µm
14 days
Crush site
C
100 µm
Control
Crush site
D
50 µm

Figure 4.11: 12 µm thick frozen transverse sections of retinas with fluorogold (labelling RGCs in gold) (A) 4 days after optic nerve crush, **(B)** 7 days after optic nerve crush, **(C)** 14 days after optic nerve crush and **(D)** no optic nerve crush. There is a loss of retinal ganglion cells over time after optic nerve crush.

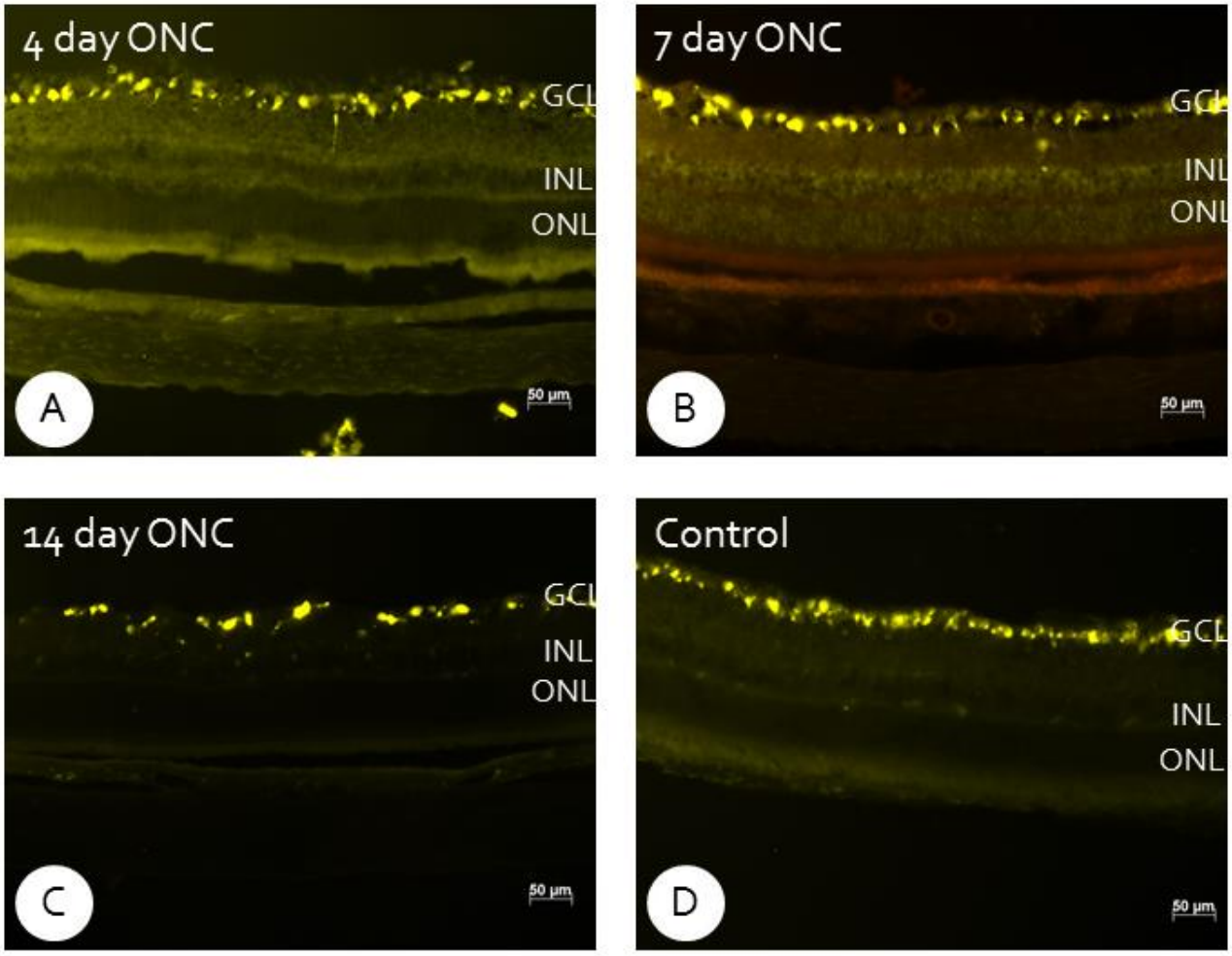

4 day ONC
GCL
INL
ONL
A
50 µm
7 day ONC
GCL
INL
ONL
B
50 µm
14 day ONC
GCL
INL
ONL
C
50 µm
Control
GCL
INL
ONL
D
50 µm

Figure 4.12: RGC survival 14 days after intravitreal injection of HAPI cells. There was no significant change from control (black line) when HAPI cells were injected without ONC (red) until 14 days after. The RGC density was lower after HAPI cells were injected with ONC (black triangles) when compared to ONC alone in the first 7 days. After 14 days of injection and ONC, there was no significant different from ONC alone.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

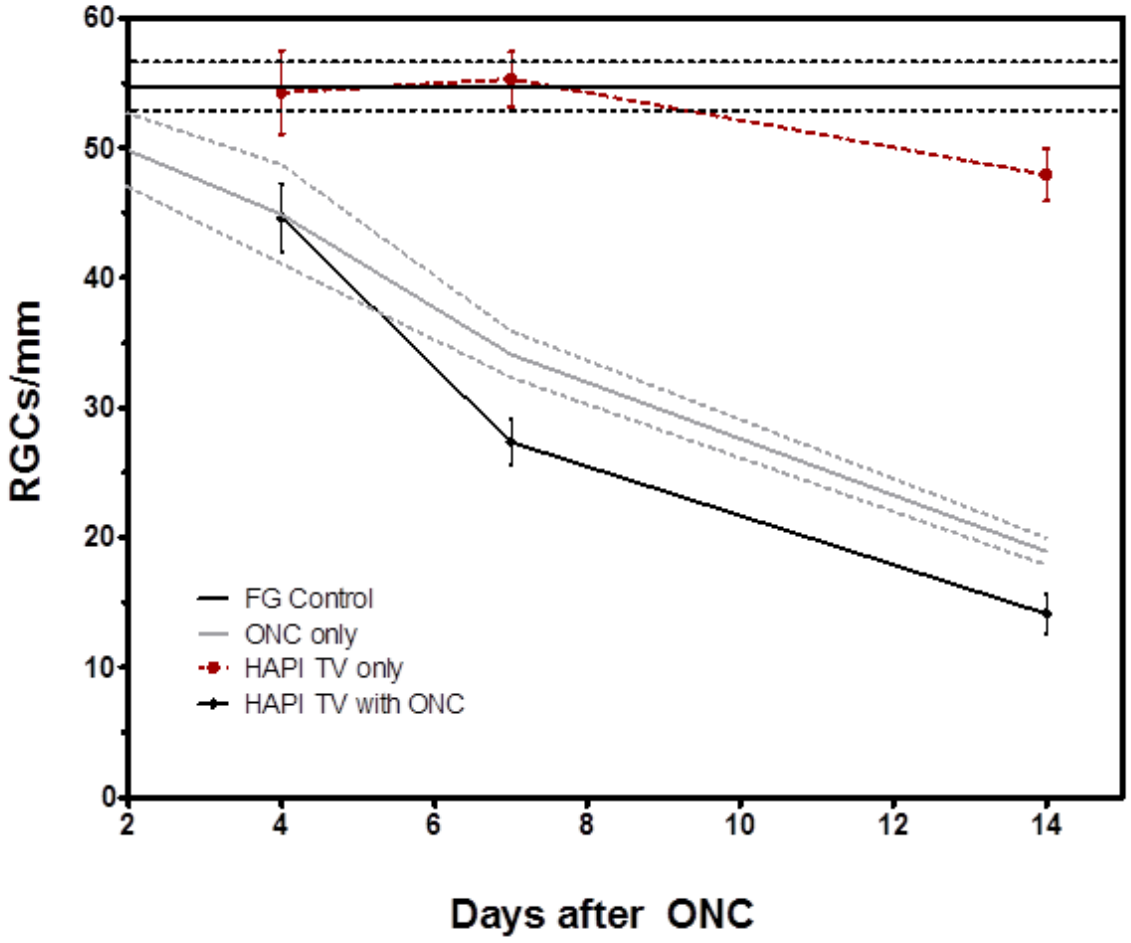
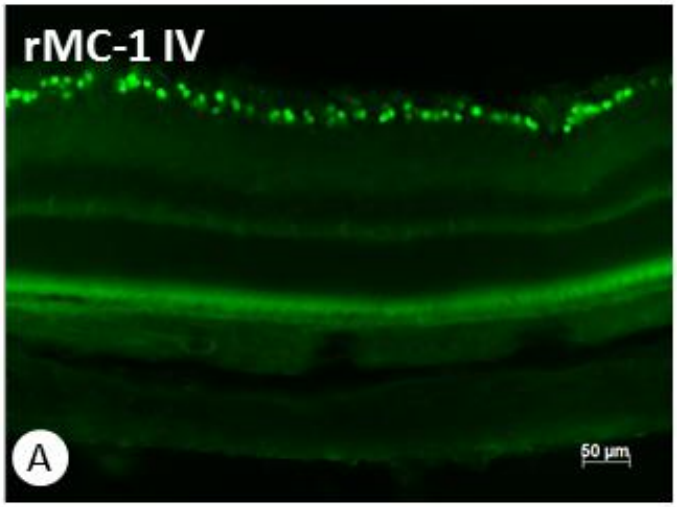
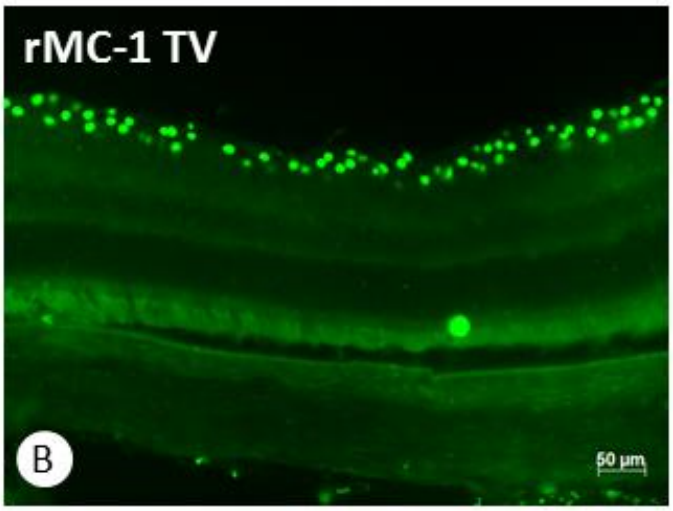

RGCs/mm
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
FG Control
ONC only
HAPI TV only
HAPI TV with ONC

Figure 4.13: 12 µm thick frozen transverse sections of retinas after injection of rMC-1 cells. (A) Retinal micrographs look similar to control after rMC-1 injection into the vitreous. **(B)** Retinal micrographs look similar to control after rMC-1 injection into the tail vein. **(C)** Retinal micrograph of controls. RGCs are labeled with Brn3b (green).


rMC-1 IV
A
50 µm


rMC-1 TV
B
50 µm

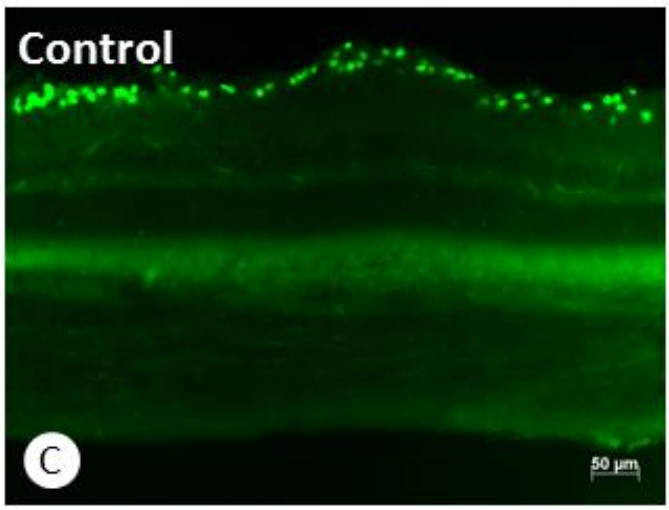

Control
C
50 µm

Figure 4.14: RGC survival 14 days after intravitreal or tail vein injection of rMC-1 cells. There is no significant RGC loss when rMC-1 cells are injection into the vitreous (blue) or tail vein (red) when compared to control (grey).

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

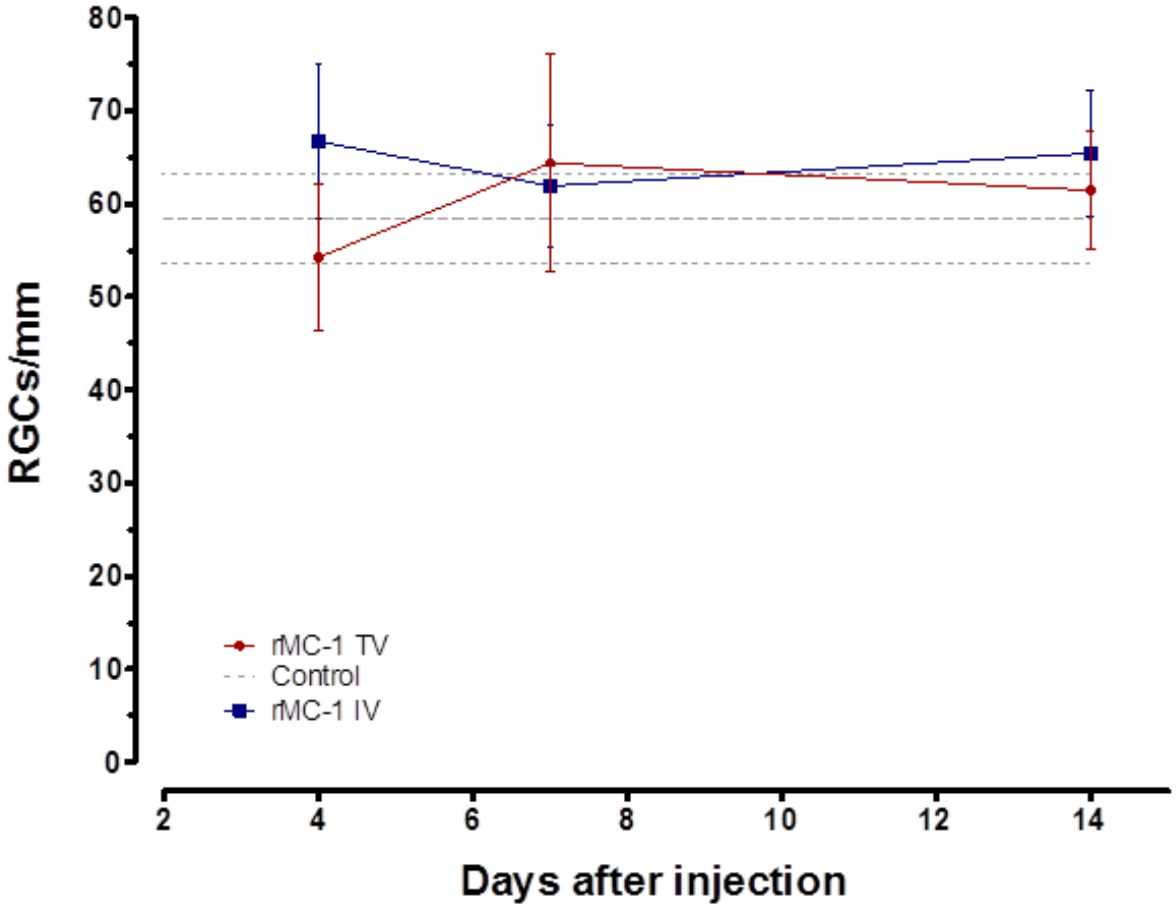

RGCs/mm
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after injection
rMC-1 TV
Control
rMC-1 IV

Figure 4.15: RGC survival 14 days after tail vein injection of HAPI cells.

There was no significant change from control (black line) when HAPI cells were injected without ONC (red) until 14 days after injection. The RGC density was lower after HAPI cells were injected with ONC (black circle) when compared to ONC alone 7 days after the injury. Early in the injury, tail vein injected resulted in no additional loss of RGC than expected and ONC alone.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

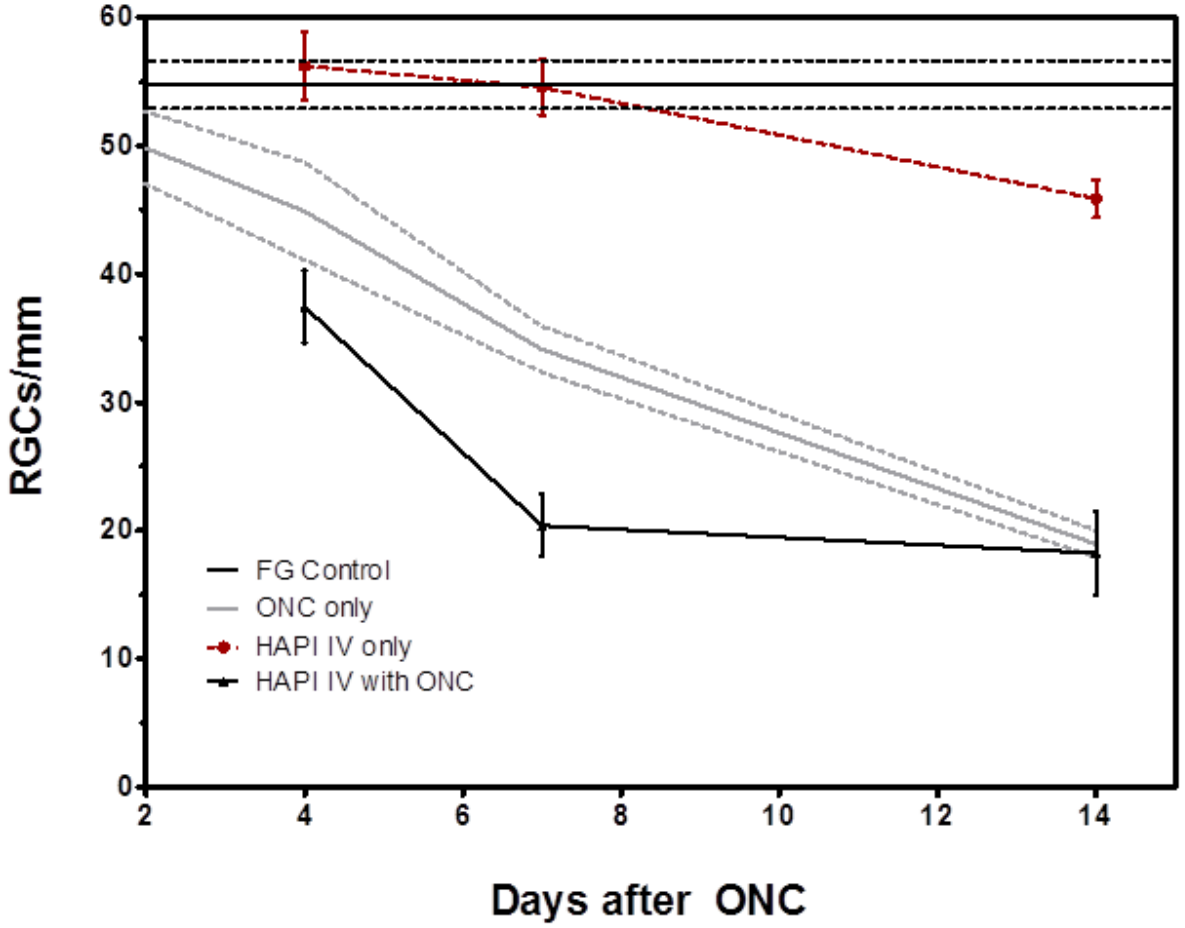
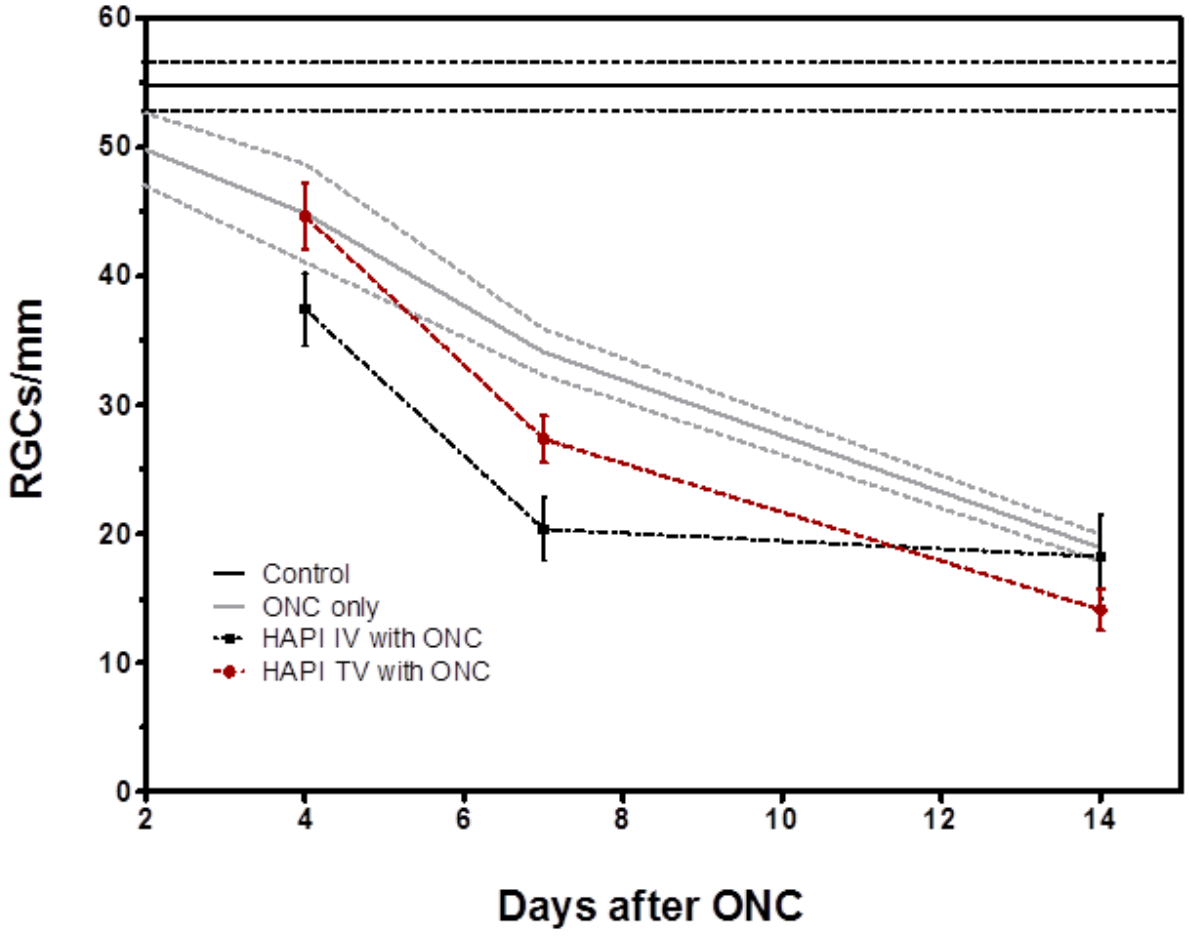

RGCs/mm
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
FG Control
ONC only
HAPI IV only
HAPI IV with ONC

Figure 4.16: RGC survival 14 days after injection of HAPI cells. The RGC density 7 days after tail vein injection and ONC (red) was intermediate to that of ONC alone (black circle) and after HAPI cell injection in the vitreous followed by ONC (grey).

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


RGCs/mm
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
Control
ONC only
HAPI IV with ONC
HAPI TV with ONC

CHAPTER 5: DOWN-REGULATION OF BM88 AFTER OPTIC NERVE INJURY

5.1 Results

BM88 has been used as a reliable marker of RGCs after optic nerve injury because its expression would not be expected to change due to its involved in maintaining cell cycle arrest and RGCs are post-mitotic (Wakabayashi, Fukuda, & Kosaka, 1996a, 1996b; Wakabayashi et al., 2010). Thy1.1 has also been used as a marker of RGCs to evaluate their loss after transection (Kielczewski, Pease, & Quigley, 2005). However, after optic nerve crush a decrease in thy1 expression preceded the loss of RGCs (Huang, Fileta, Guo, & Grosskreutz, 2006; Schlamp, Johnson, Li, Morrison, & Nickells, 2001). This suggests that there is a difference in the expression of RGC markers depending on the type of injury. The purpose of this study was to determine if BM88 expression was correlated with RGC loss after optic nerve crush (ONC) in young adult rats. An overview of this chapter can be found in Appendix II.

5.1.1 RGC Loss after Optic Nerve Injury

There was an expected gradual loss of FG containing RGCs after optic nerve crush (ONC). In the absence of an injury, there were 56.46 ± 3.09 RGCs/mm (n=6; figure 5.1 and 5.2). The RGC density 2 days after ONC was 53.06 ± 0.92 RGCs/mm, which was not significantly different from the control (n=4; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p > 0.05$). The RGC density 4 days after ONC was significantly lower with 47.96 ± 3.53 RGCs/mm remaining (n=6; figure 5.1 and 5.2; ANOVA/Tukey's; $p \leq 0.001$). This represented a loss of 15.1% of RGCs compared to uninjured retinas. At 7 days

after ONC there were 30.42 ± 2.15 RGCs/mm remaining, representing a loss of 46.1% of RGCs compared to controls (n=6; figure 5.1 and 5.2; ANOVA/Tukey's; $p \leq 0.001$). The RGC density after 14 days of ONC was 17.61 ± 1.38 RGCs/mm (n=6; figure 5.1 and 5.2; ANOVA/Tukey's; $p \leq 0.001$). The RGC density after 28 days of ONC was 11.15 ± 4.93 RGCs/mm, representing a loss of 80.3% of the RGCs present in the control (n=4; figure 4.1 and 4.2; ANOVA/Tukey's; $p \leq 0.001$).

A similar loss of RGCs occurred after the optic nerve transection (ONT) injury. There was no significant difference in cell count 2 days after ONT as there were 58.22 ± 7.07 RGCs/mm (n=4; figure 5.3 and 5.4; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, there was a significant difference 4 days after ONT with 48.40 ± 4.69 (n=4; figure 5.3 and 5.4; ANOVA/Tukey's $p \leq 0.05$). At 7 days after ONT there were 30.38 ± 1.96 RGCs/mm remaining, representing a loss of 46.2% of RGCs from the control (n=4; figure 5.3 and 5.4; ANOVA/Tukey's; $p \leq 0.001$). The RGC density after 14 days of ONT was 16.18 ± 3.40 RGCs/mm. The RGC density after 28 days of ONT was 7.04 ± 3.53 RGCs/mm, representing a loss of 87.53% of the cells compared to controls (n=4; figure 5.3 and 5.4; ANOVA/Tukey's; $p \leq 0.001$).

5.1.2 Co-Localization of BM88 with Fluorogold

In non-injured retinas there were 55.85 ± 3.12 BM88 immunoreactive RGCs/mm, representing 98.9% of all RGCs (n=6; figure 5.1 and 5.2). Similarly, 2

days after ONC there were 51.39 ± 1.55 BM88 immunoreactive RGCs/mm, which was not significantly different from control (n=4; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p > 0.05$). There was no difference 4 days after ONC as there were 43.39 ± 3.39 BM88 immunoreactive RGCs/mm (n=6; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, 7 days after ONC there was a significant reduction in the number of BM88 immunoreactive RGCs, with 5.96 ± 2.08 immunoreactive RGCs/mm remaining (n=6; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p \leq 0.001$). This represented only 19.6 % of the RGCs that remained after 7 days of ONC. Similarly, 14 and 28 days after ONC there were only 2.54 ± 1.00 (n=6; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p \leq 0.001$) and 2.53 ± 2.32 (n=4; figure 5.1 and 5.2; ANOVA/Tukey's post-hoc test; $p \leq 0.01$) BM88 immunoreactive RGCs/mm remaining (respectively). This represented only 14.4 % of the RGCs that remained after 14 days of ONC and 22.7 % of the RGCs that remained after 28 days of ONC.

A similar trend was evident for BM88 immunoreactivity after ONT, except that the down-regulation began earlier. Two days after ONT there was no significant change from control with 56.22 ± 7.54 BM88 immunoreactive RGCs/mm (n=4; figure 5.3 and 5.4; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, at 4 days there was a significant reduction with 35.56 ± 6.98 BM88 immunoreactive RGCs/mm remaining (n=4; figure 5.3 and 5.4; ANOVA/Tukey's post-hoc test; $p \leq 0.01$). This represented only 73.5 % of the RGCs that remained

after 4 days of ONT. The loss of BM88 immunoreactive RGCs was more pronounced 7 days after ONT where there were only 14.49 ± 3.75 BM88 immunoreactive RGCs/mm remaining (n=4; figure 5.3 and 5.4; ANOVA/Tukey's post-hoc test; $p \leq 0.001$). This means that only 47.7% of the remaining RGCs were labelled with BM88. This remained the trend at 14 and 28 days after ONT, where there were 3.94 ± 1.5 (n=4; figure 5.3 and 5.4 ANOVA/Tukey's post-hoc test; $p \leq 0.001$) and 1.88 ± 0.92 (n=4; figure 5.3 and 5.4; ANOVA/Tukey's post-hoc test; $p \leq 0.01$) BM88 immunoreactive RGCs/mm remaining, respectively. This represented only 24.4 % of the RGCs that remained after 14 days of ONT and 25.6 % of the RGCs that remained after 28 days of ONT.

5.1.3 Staining Intensity of BM88

There were not only fewer RGCs labelled with BM88 after ONC but the intensity of the cells labelled was also diminished. ImageJ was used to calculate the mean grey value of the BM88 immunoreactive RGCs taken at the same exposure and then expressed as a percentage staining of the control. Initially, 2 and 4 days after ONC there was a significant increase of 19.0 ± 3.6% (n=4; figure 5.5 and 5.6; ANOVA/Tukey's; $p \leq 0.001$) and 23.7 ± 5.4% (n=6; figure 5.5 and 5.6; ANOVA/Tukey's; $p \leq 0.001$), in staining intensity, respectively. However, 7 days after ONC there was a sharp decline in staining intensity to 55.28 ± 5.24% of the control (n=6; figure 5.5 and 5.6; ANOVA/Tukey's; $p \leq 0.001$). This trend persisted for 28 days after ONC, where the staining intensity was 41.07 ± 3.76% of the control after 14 days of ONC and 38.43 ± 3.95% (n=6; figure 5.5 and 5.6;

ANOVA/Tukey's; $p \leq 0.001$) of the control after 28 days of ONC (n=4; figure 5.5 and 5.6; ANOVA/Tukey's; $p \leq 0.001$).

A similar trend was seen after ONT, except that the decrease in staining intensity occurred after 4 days of ONT instead of 7 days, as seen after ONC. Early in the injury, there was a significant increase of 7.9 ± 2.7% in staining intensity after 2 days following ONT (n=4; figure 5.7 and 5.8; ANOVA/Tukey's; $p \leq 0.001$). Then beginning 4 days after ONT, there was a decline in staining intensity where it is 54.74 ± 2.51% of the control (n=4; figure 5.7 and 5.8; ANOVA/Tukey's; $p \leq 0.001$). This trend persisted where the staining intensity was 49.33 ± 1.99% of the control 7 days after ONT, 55.34 ± 5.29% of the control 14 days after ONT, and 51.04 ± 11.65% of the control 28 days after ONT (n=4; figure 5.7 and 5.8; ANOVA/Tukey's; $p \leq 0.001$).

5.2 Discussion

The ganglion cell layer (GCL) of the retina contains both RGCs and displaced amacrine cells in approximately equal proportions (Jeon, Strettoi, & Masland, 1998; Perry, 1981). This makes it difficult to perform quantitative analyses of RGC loss, because a measure of total cell number will underestimate RGC loss due to the large number of uninjured displaced amacrine cells. The standard for labelling RGCs has been to use retrogradely transported tracers injected into the superior colliculus, such as fluorogold (Koeberle & Ball, 1998; Salinas-Navarro et al., 2009). The use of antibodies has been attractive because it is less labour intensive and reduces the amount of invasive procedures performed

on the animals. One such antibody that has been used for this purpose is Thy1.1. The issue with Thy1.1 is that its expression pattern changes after injury (Huang et al., 2006; Schlamp et al., 2001). Initially BM88 seemed to be a good marker for non-injured RGCs because it labelled nearly all FG labelled cells and no displaced amacrine cells. However, 4-7 days after optic nerve injury, the number of BM88 immunoreactive RGCs started to decrease. The staining intensity decreases earlier in ONT than ONC (4 vs. 7 days) because an ONT is a more serve injury (Villegas-Pérez, Vidal-Sanz, Rasminsky, Bray, & Aguayo, 1993) and, therefore, intracellular changes may occur sooner. At 7 days after optic nerve injury there were only 20-50% of RGCs still immunoreactive for BM88 and of these cells the expression of BM88 (as measured by staining intensity) was 49-55% of the control. This trend persisted 14 and 28 days after optic nerve injury. Therefore, BM88 is not a good marker for recording numbers of remaining RGCs after injury, however, changes in its expression may be a useful marker of abnormal RGC function that precedes RGC loss.

We have shown that after optic nerve injury there was a reduction in the number of RGCs immunoreactive for BM88 and that there was a reduction in the staining intensity of the RGCs that still express BM88. However, early in the injury there was an increase in BM88 staining intensity. Four days after ONC and 2 days after ONT there was an increase of 20% and 7.9% (respectively) in staining intensity compared to the control. This suggests that the early changes in the physiological health of the RGCs before the death of the cell can be revealed

by BM88 expression. This initial increase may be an abortive attempt by the cell to survive after the injury. An increase in BM88 expression may help reduce calcium mobilization by increasing calcium uptake by the mitochondria and lowering the calcium storage in the endoplasmic reticulum (Masgrau et al., 2009). Experiments using BM88 overexpressing neuroblastoma cells show that BM88 may have a neuroprotective role against neurotoxic stimuli through the inhibition of ERK and BAX (Politis, Thomaidou, & Matsas, 2008).

The down-regulation of such an important suppressor of entry into the cell cycle raises many questions. It could suggest that RGCs, after axonal injuries, may attempt to re-enter the cell cycle which leads to cell death. Cell cycle molecules have been shown to be involved in normal developmentally associated neuronal death (Becker & Bonni, 2004; Greene, Liu, Troy, & Biswas, 2007). In normal development, approximately 50% of neurons undergo cell death (Oppenheim, 1991). Neuronal survival also heavily relies on competition for target-derived trophic factors (Ernfors, Lee, & Jaenisch, 1994; Klein, 1994; Ma, Hsieh, Forbes, Johnson, & Frost, 1998; Sendtner, Pei, Beck, Schweizer, & Wiese, 2000). After loss of the synaptic target of postmitotic cerebellar granule cells resulted in cell death that was associated with elevation of cyclin D and other cell cycle proteins (Herrup & Busser, 1995). It seems that G1/S related events are required for neuronal death in development. When the transition to this phase was blocked, it rescued neurons from cell death due to NGF deprivation (Farinelli & Greene, 1996; Ferrari & Greene, 1994). Deprivation of nerve growth factor in

sympathetic neuron cultures was associated with an increase in transcripts encoding cyclin D1 (Freeman, Estus, & Johnson Jr, 1994). BM88 is an important inhibitor of cyclin D1 and may play a role in regulating apoptosis of neurons. Neurotrophic deprivation is also thought to play an important role in glaucoma (Quigley et al., 2000; Van Adel, Arnold, Phipps, Doering, & Ball, 2005; Vrabec & Levin, 2007) and, therefore, apoptosis after neurotrophic deprivation may be regulated by cell cycle molecules, such as BM88 and cyclin D1. There are examples of neurons re-entering the cell cycle in other neurodegenerative disorders, such as Alzheimer's disease (Raina et al., 2000). This type of dysregulation of the cell cycle is known to put neurons into an apoptosis prone state (Politis, Thomaidou, & Matsas, 2008(Bonda et al., 2009)). Cell cycle related proteins are found to be elevated in neurons that are at risk to die in many different neurodegenerative disorders (Nguyen et al., 2003; Ogawa et al., 2003). Cyclin D1 re-expression is observed in patients with neurodegenerative diseases, such as Alzheimer's disease (Raina et al., 2000). In addition, cyclin D1 is expressed in high levels in lesioned hippocampal neurons suggesting that BM88 acts as a modulator of apoptosis (Timsit et al., 1999). All this taken together implicates BM88 as an important suppressor of the cell cycle in mature neurons and its dysregulation may cause cells to be more prone to apoptosis. Future studies should examine the interaction between BM88 down-regulation and other proteins involved in the cell cycle (such as cyclin D1) and the effects of maintaining BM88 expression on RGC survival.

BM88 is also thought to play roles in calcium buffering and helping maintain the integrity of the mitochondria (Masgrau et al., 2009). Calcium regulation in neurons is important because calcium overload in the cytoplasm and mitochondria leads to apoptosis (Orrenius, Zhivotovsky, & Nicotera, 2003). BM88 acts to block calcium release from calcium stores in the cell by modulating IP_3 and IP_3R (Masgrau et al., 2009). Down regulation of BM88 in RGCs may cause the release of calcium from intracellular stores leading to apoptosis of the cell. Blocking voltage dependent calcium channels with Flunarizine has down to enhance RGC survival after axotomy in rats (Eschweiler & Bähr, 1993). BM88 is also found on the membrane of mitochondria but the role it has there is not well understood (Georgopoulou et al., 2006). However, it is thought that BM88 inhibits ERK leading to BAX not being able to bind to the mitochondria (Masgrau et al., 2009). When BAX binds to the mitochondria, this initiates the pathway for apoptosis (Wei et al., 2001). Therefore, there are 3 mechanisms by which down regulation of BM88 may lead to RGC cell death: (1) impaired calcium regulation, (2) initiation of apoptosis and mitochondrial dysfunction, and (3) cell cycle dysregulation.

5.3 Figures and Tables

Figure 5.1: Down-regulation of BM88 after optic nerve crush (ONC). Before injury, most fluorogold (gold) labelled RGCs co-localize with BM88 (red). After injury, there is a decrease in the number of RGCs immunoreactive for BM88 before the death of the RGCs themselves.

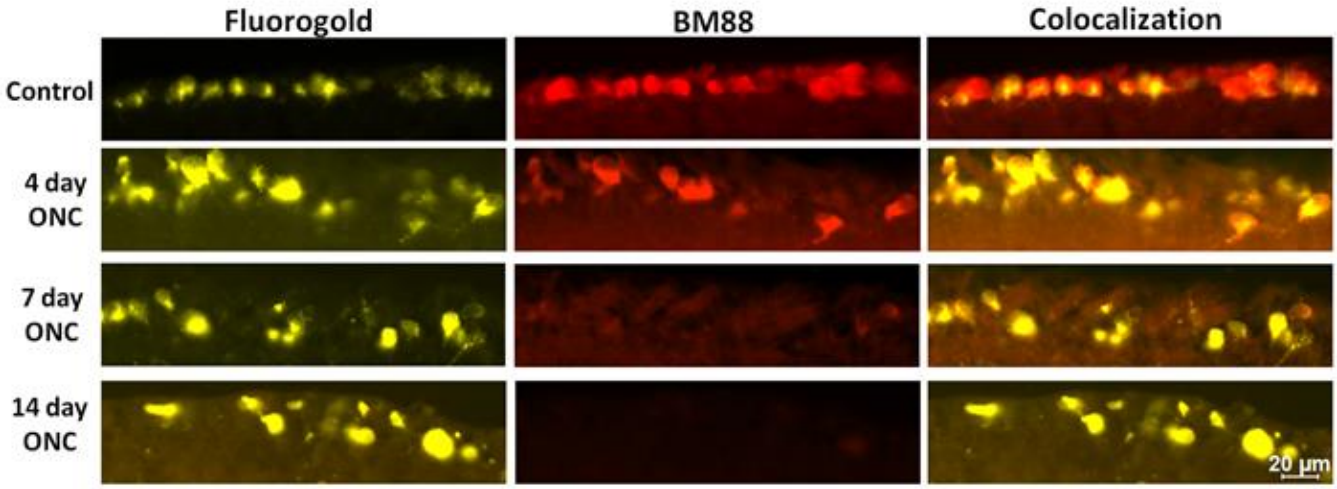

Fluorogold
BM88
Colocalization
Control
4 day ONC
7 day ONC
14 day ONC
20 µm

Figure 5.2: The decrease in BM88 immunoreactive RGCs preceded the loss of RGCs following optic nerve crush (ONC). In control retinas, 99% of BM88 immunoreactive RGCs were co-localized with FG. However, there were only 20% of RGCs immunoreactive for BM88 7 days after ONC.

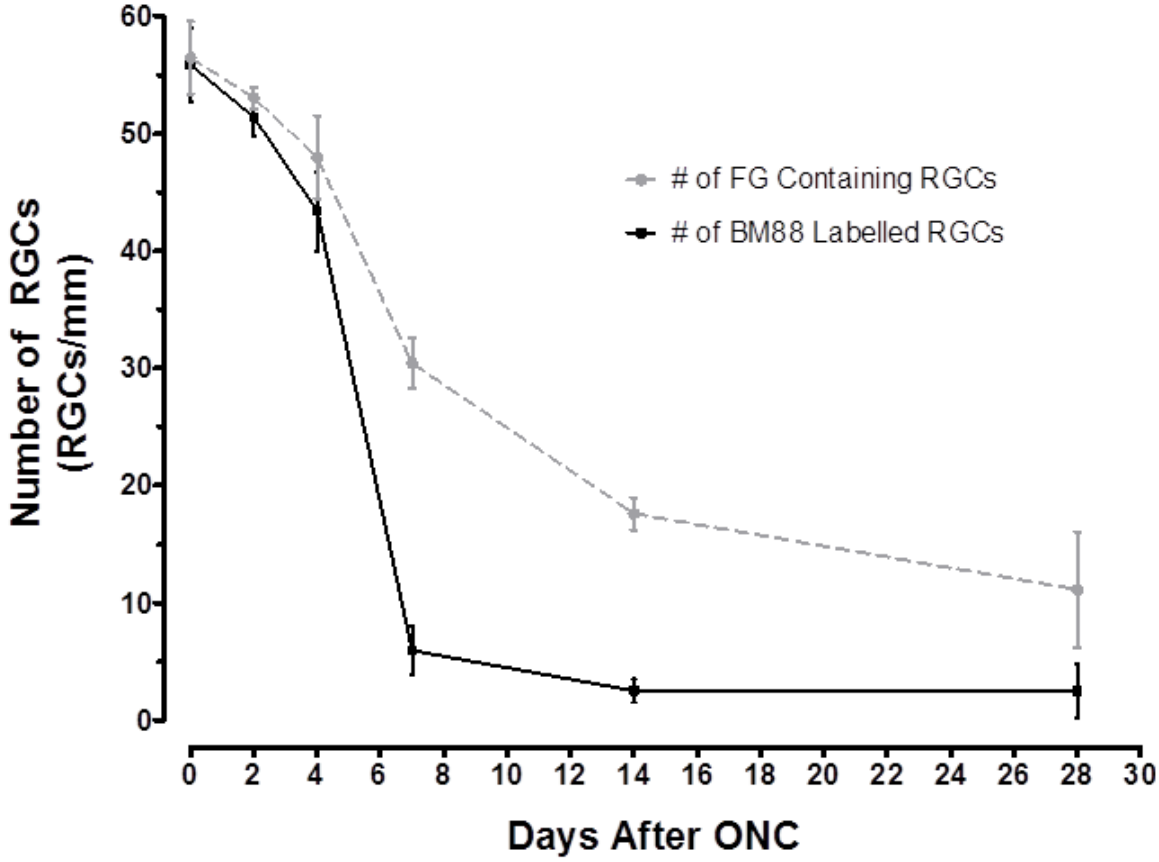

Number of RGCs
(RGCs/mm)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days After ONC
# of FG Containing RGCs
# of BM88 Labelled RGCs

Figure 5.3: Down-regulation of BM88 after optic nerve transection (ONT).

Before injury, most fluorogold (gold) labelled RGCs co-localize with BM88 (red). After injury, there is a decrease in the number of RGCs immunoreactive for BM88 before the death of the RGCs themselves.

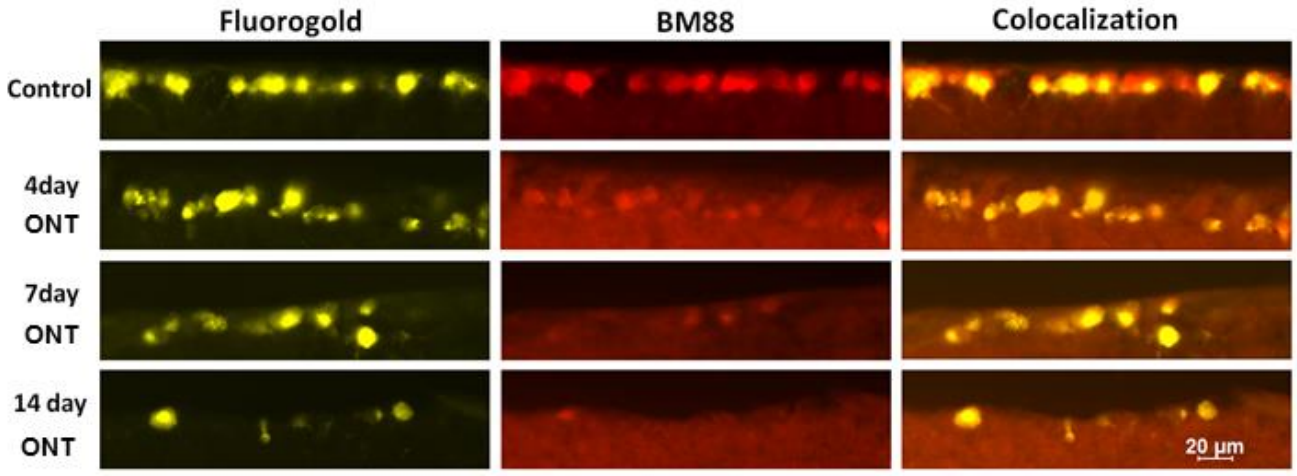

Fluorogold
BM88
Colocalization
Control
4day
ONT
7day
ONT
14 day
ONT
20 µm

Figure 5.4: The decrease in BM88 immunoreactive RGCs preceded the loss of RGCs following optic nerve transection (ONT). In control retinas, 99% of BM88 immunoreactive RGCs were co-localized with FG. However, there were only 48% of RGCs immunoreactive for BM88 7 days after ONT.

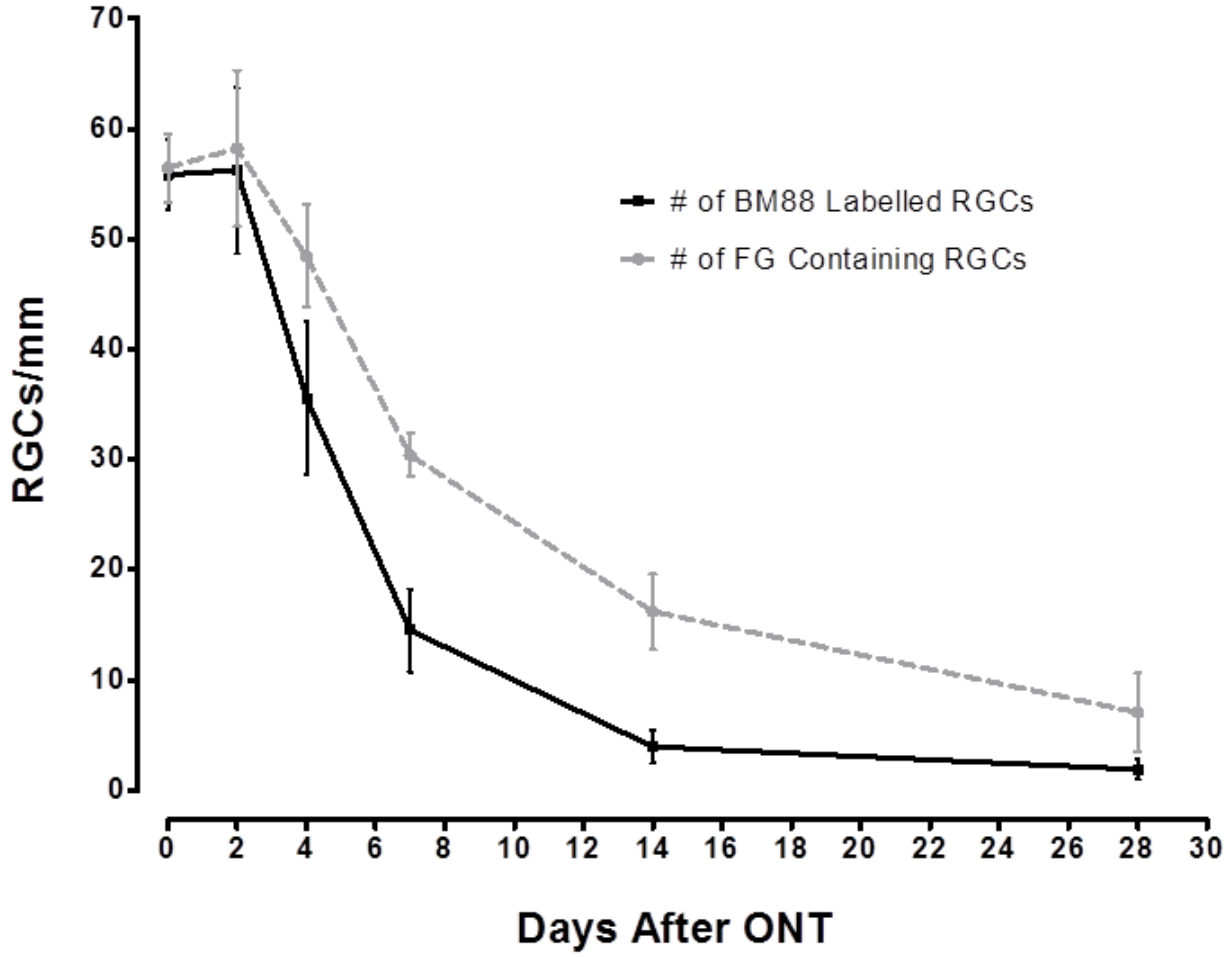
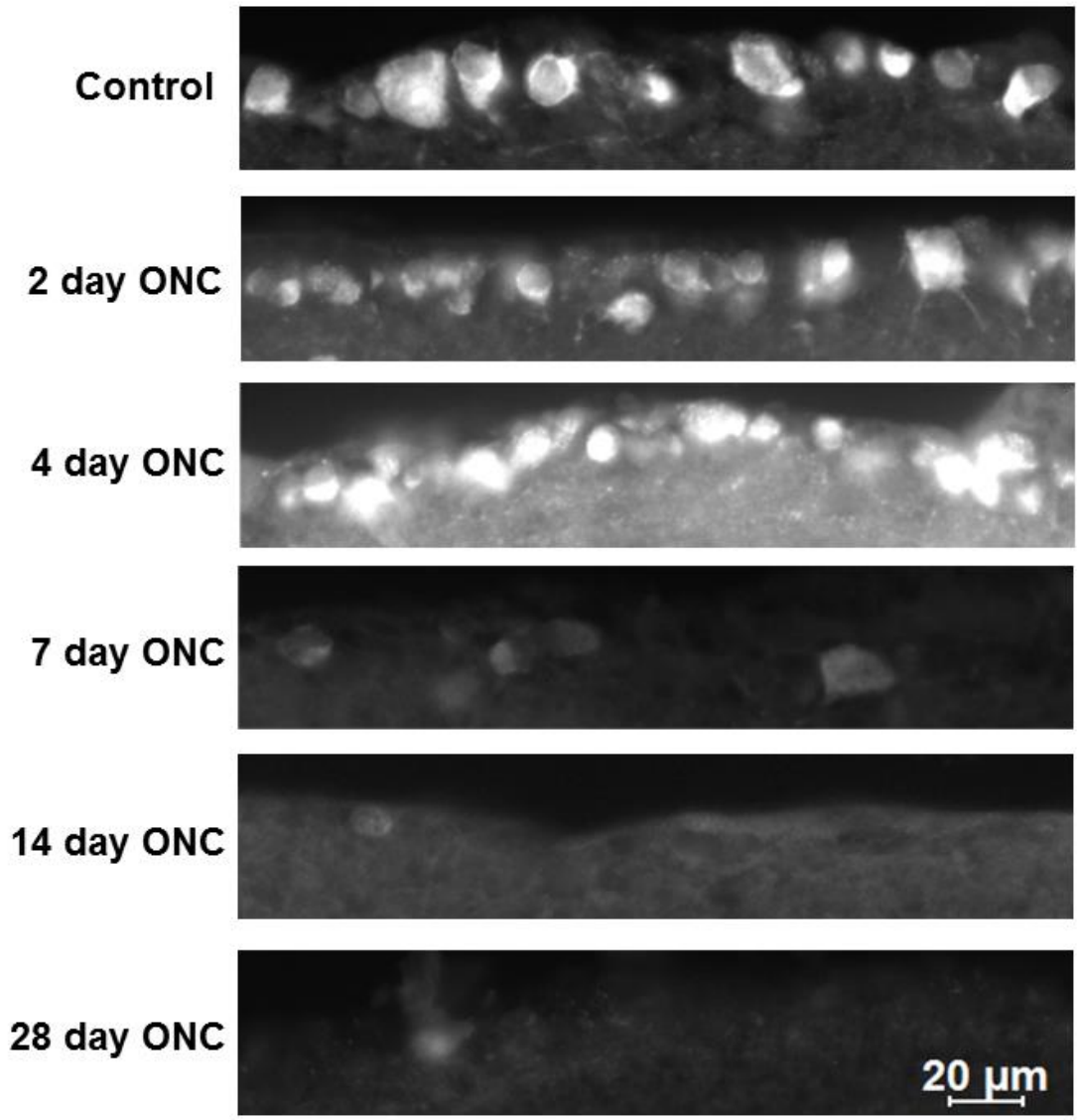

70
60
50
40
30
20
10
0
RGCs/mm
# of BM88 Labelled RGCs
# of FG Containing RGCs
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days After ONT

Figure 5.5: Down-regulation of BM88 after optic nerve crush (ONC). Before injury, most RGCs co-localized with BM88. Early in the injury (at 4 days), there was an increase in the staining intensity of BM88. After 7 days of injury, there was a decrease in the number of RGCs immunoreactive for BM88 and a decrease in the staining intensity of BM88 before the loss of the RGC itself.


Control
2 day ONC
4 day ONC
7 day ONC
14 day ONC
28 day ONC
20 µm

.

Figure 5.6: Decrease in staining intensity of BM88 after optic nerve crush (ONC). Four days after injury there was an increase of 24% in the staining intensity of BM88. However, after 7 days of injury, there was a 55% decrease in the staining intensity of BM88. From 14 days of injury the staining intensity dropped to its lowest point and this remained constant over the 28 day period.

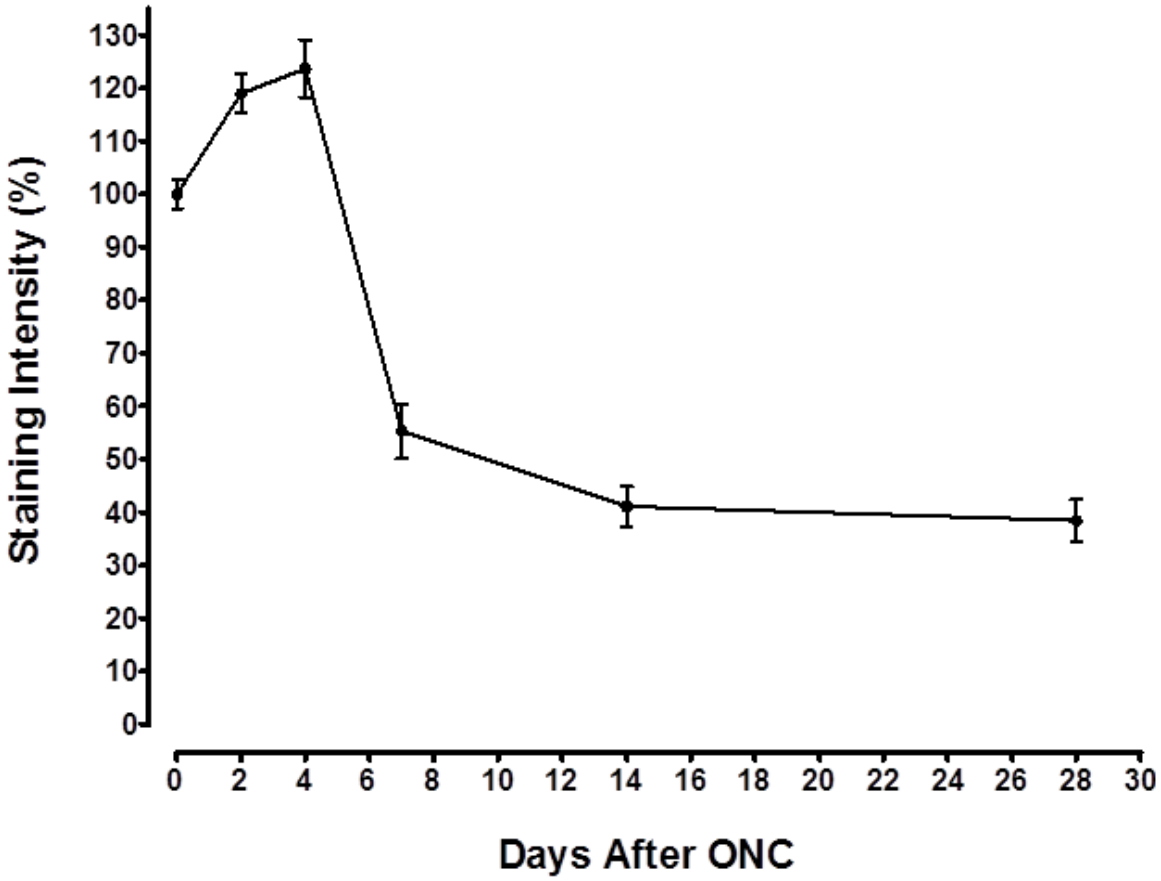

Staining Intensity (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days After ONC

Figure 5.7: Down-regulation of BM88 after optic nerve transection (ONT). Before injury, most RGCs co-localized with BM88. Two days after injury there was an increase in the staining intensity of BM88. This was earlier than that seen after ONC. After 4 days of injury, there was a decrease in the number of RGCs immunoreactive for BM88 and a decrease in the staining intensity of BM88 before the loss of the RGC itself.

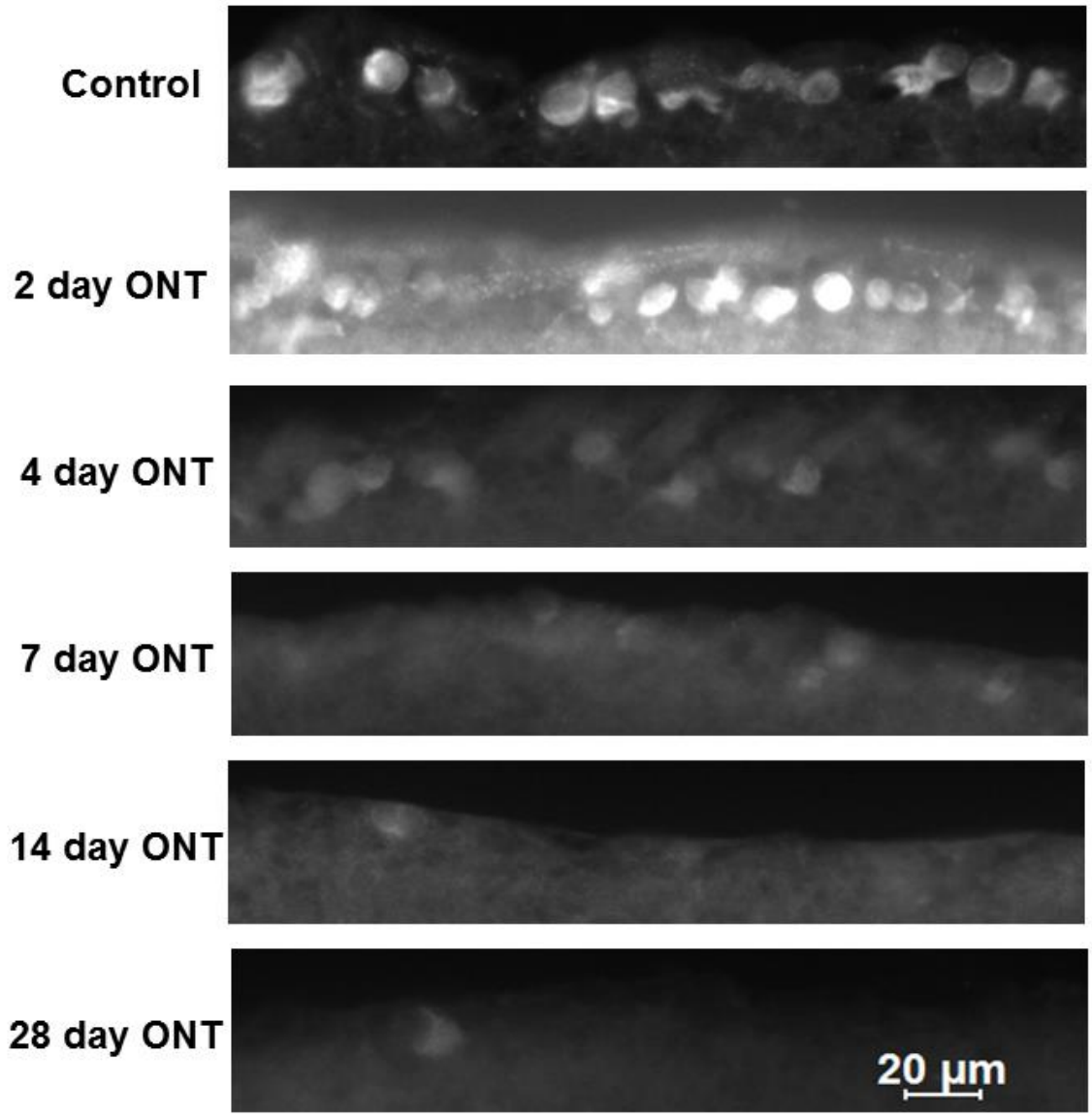
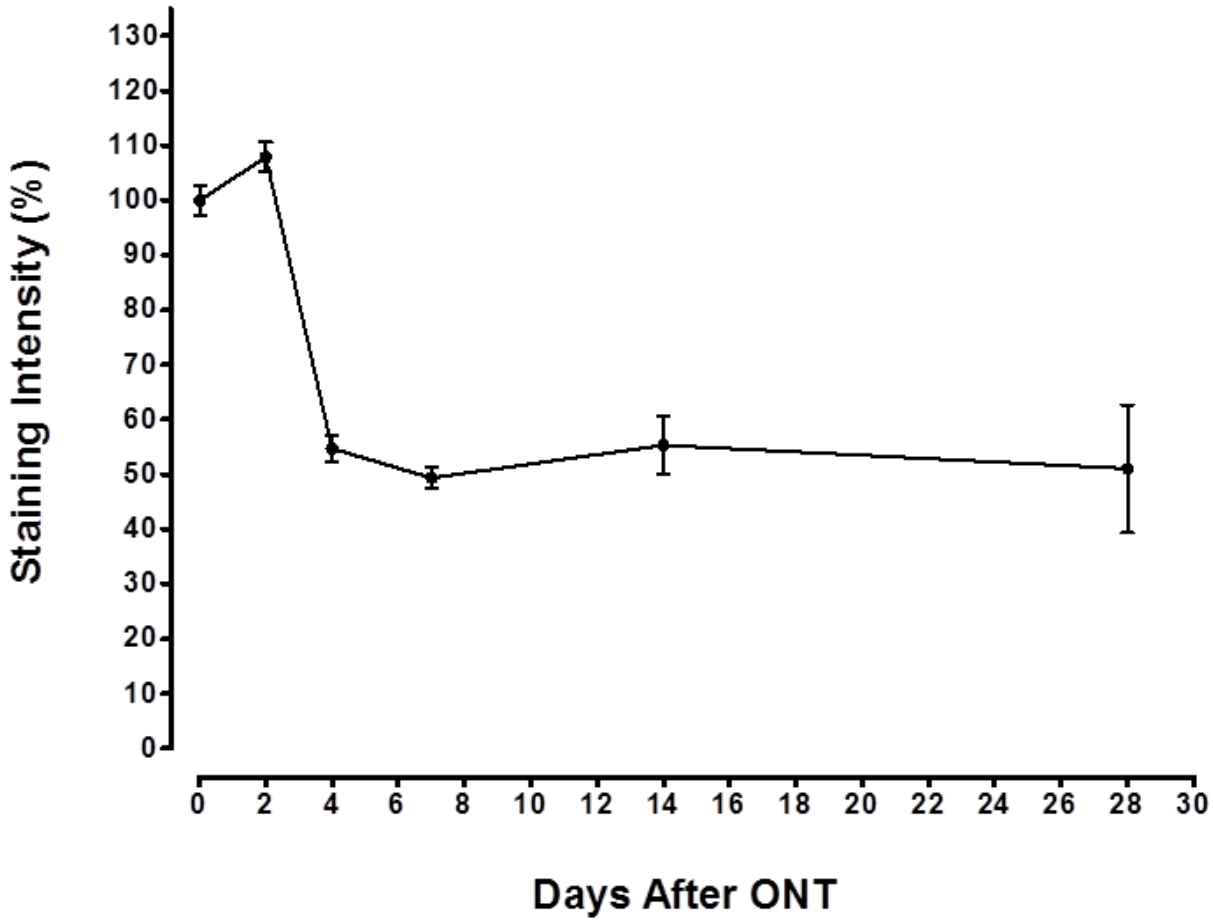

Control
2 day ONT
4 day ONT
7 day ONT
14 day ONT
28 day ONT
20 µm

Figure 5.8: Decrease in staining intensity of BM88 after optic nerve transection (ONT). Two days after injury there was an increase of 8% in the staining intensity of BM88. However, after 4 days of injury, there was a 55% decrease in the staining intensity of BM88 and this remained constant over the 28 day period.


Staining Intensity (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days After ONT

CHAPTER 6: EFFECT OF MICROGLIAL ACTIVATION ON BM88 EXPRESSION IN RETINAL GANGLION CELLS

6.1 Results

It was suggested in chapter 5 that changes in expression of BM88 may be indicative of abnormal retinal ganglion cell (RGC) functioning after injury. We have previously shown that when cultured HAPI microglial cells were injected into the eye or tail vein that it resulted in a pro-inflammatory response leading to greater RGC loss (chapter 4). The purpose of this study was to determine if a pro-inflammatory environment produced by HAPI cells would lead to BM88 expression changes in injured RGCs. It was my hypothesis that since there was greater RGC loss after HAPI cell injection that there would be earlier down regulation of BM88 in RGCs. This would also suggest that cytotoxic neuroinflammation leads to cell cycle dysregulation, mitochondrial dysfunction, and/or impaired calcium regulation in RGCs that ultimately leads to apoptosis. An overview of this chapter is in Appendix III.

6.1.1 RGCs Survival after Intravitreal Injection of HAPI Cells

In the absence of an injury, there were 58.40 $\pm$ 4.71 RGCs/mm (n=7). Our previous results show that there was no statistical difference between the RGC density in the control retinas and when HAPI cells were injected into the vitreous without injury (Chapter 4). This was replicated here with Brn3a immunostaining of RGCs. Four days after injection of HAPI cells into the vitreous without ONC, there were 54.30 ± 2.14 RGCs/mm remaining (n=6; figure 6.1 and 6.3). This was also not significantly different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$). Similarly, there was a RGC density of 52.33 ± 2.82 RGCs/mm 7

days after HAPI cells were injected into the vitreous. This was not significantly different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$). The trend that was presented in Chapter was replicated here where there was RGC loss 14 days after HAPI cells were injected into the vitreous in the absence of injury. The RGC density was 50.58 ± 3.39 RGCs/mm 14 days after HAPI cell injection into the vitreous. This was 13.4% less than the RGC density of controls (n=7; ANOVA/Tukey's post-hoc test, $p \leq 0.01$).

Our previous results (Chapter 4) also demonstrated a loss of RGCs when HAPI cells were injected into the vitreous when accompanied by an ONC. This finding was also replicated here with Brn3a staining. The RGC density was 35.55 ± 2.47 RGCs/mm 4 days after HAPI cell injection into the vitreous followed by ONC (n=6; figure 6.2 and 6.3). This represented a loss of 20.8% more RGCs than would be expected to be lost after ONC alone (n=7; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). After 7 days following the injection of HAPI cells into the vitreous accompanied by an ONC there were 22.23 ± 2.06 RGCs/mm remaining (n=6). This were 34.9% more RGCs lost than would be expected after ONC alone (n=7; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). The RGC density was 17.08 ± 2.86 RGCs/mm 14 days after HAPI cell were injected into the vitreous with an ONC injury (n=6). This was not significantly different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$).

6.1.2 Co-localization of BM88 with Brn3a after Intravitreal Injection of HAPI Cells

We have previously shown that there was no statistical difference between the RGC densities in control retinas and RGCs labelled with BM88 (Chapter 5). Control retinas had 57.53 ± 3.51 RGCs/mm expressing BM88 (n=7; Figure 6.1 and 6.4). This was not different from the number of RGCs expressing Brn3a (figure 6.6; n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$). There were 48.41 ± 4.46 RGCs/mm expressing BM88 4 days after HAPI cell injection into the vitreous without ONC (n=6). This was not significantly different from the number of RGCs expressing Brn3a 4 days after HAPI cell injection into the vitreous (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$; figure 6.5). However, after 7 days following the injection of HAPI cells into the vitreous and without injury there were 41.22 ± 5.3 RGCs/mm expressing BM88. This was 28.1% less than the number of RGCs expressing Brn3a 7 days after HAPI cell injection into the vitreous (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). Similarly, there were 46.10 ± 6.7 RGCs/mm expressing BM88 14 days after HAPI cells were injected into the vitreous (n=6). This was 19.9% less than the BM88 immunoreactive RGCs in controls but was not different from the number of Brn3a expressing RGCs 14 days after HAPI cells were injected into the vitreous.

Previous results (from chapter 5) showed that only 19.6% of RGCs expressed BM88 7 days after injury but was the same as in control retinas 4 days after injury. After 4 days following injecting HAPI cells into the vitreous

accompanied by an ONC there were 23.60 ± 3.61 RGCs/mm expressing BM88 (n=6; figure 6.4). This was 33.6% less RGCs than those that expressed Brn3a 4 days after HAPI cells were injected into the vitreous accompanied by an ONC (n=6; figure 6.5; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 44.6% lower than the number of RGCs that expressed BM88 4 days after ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). There were 9.47 ± 2.97 RGCs/mm (n=6) that expressed BM88 7 days after injection of HAPI cells into the vitreous accompanied by an ONC and 2.74 ± 1.86 RGCs/mm after 14 days (n=6) . This was 57.4% fewer RGCs than those that expressed Brn3a 7 days after injection accompanied by an ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$) and 84.0% fewer RGCs 14 days later (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). The number of RGCs expressing BM88 7 and 14 days after HAPI cell injection and ONC was not statistically lower than the number of RGCs that expressed BM88 after ONC alone (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$).

6.1.3 RGCs Survival after Tail Vein Injection of HAPI Cells

Our previous results (Chapter 4) showed that there was a 20-25% loss of RGCs after HAPI cells were injected into the tail vein after ONC and no loss in the absence of crush. There were 52.21 ± 3.13 RGCs/mm when HAPI cells were injected into the tail vein without any injury to the optic nerve (n=6; figure 6.6 and 6.8). This was not statistically different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$). After 7 days of injecting the HAPI cells (n=6), the RGC density was not significantly different from controls (n=7) and remained at 54.06

± 2.72 RGCs/mm (ANOVA/Tukey's post-hoc test, $p > 0.05$). Similar to our previous results using fluorogold, there was a 15.6% RGC loss 14 days after HAPI tail vein injection as compared to the controls (n=7; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). There were 49.27 ± 2.23 RGCs/mm remaining 14 days after HAPI cell injection into the tail vein (n=6).

The RGC survival after HAPI cells were injected into the tail vein accompanied by an ONC was similar whether evaluated using fluorogold retrograde labelling (Chapter 5) or Brn3a immunohistochemistry. After 4 days following injection of HAPI cells into the tail vein followed by ONC there were 42.54 ± 1.95 RGCs/mm (n=6; figure 6.7 and 6.8). This was not statistically different from ONC alone (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, 7 days after injection of HAPI cells into the tail vein and ONC there were 23.03 ± 3.18 RGCs/mm remaining (n=6). This represents a loss of 32.5% more cells than would be expected after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). The RGC survival after 14 days of injection (n=6) and ONC (n=6) was not statistically different from the RGC density 14 days after ONC alone, where it remained at 17.13 ± 2.24 RGCs/mm (ANOVA/Tukey's post-hoc test; $p > 0.05$).

6.1.4 Co-localization of BM88 with Brn3a after Tail Vein Injection of HAPI Cells

A similar down regulation of BM88 expression by RGCs was observed after tail vein injection of HAPI cells, as it was after intravitreal injection of HAPI cells. There were 41.49 ± 5.04 RGCs/mm immunoreactive for BM88 (n=6; figure

6.6 and 6.9) 4 days after HAPI cell injection, which was 20.5% fewer than the number of RGCs immunoreactive for Brn3a (n=6; figure 7.10; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). This was statistically the same as the decrease in the number BM88 expressing RGCs after 4 days of ONC (n=6; ANOVA/Tukey's post-hoc test). There was no statistically significant difference between the RGC density of the Brn3a expressing RGCs and BM88 expressing RGCs 7 and 14 days after injection of HAPI cells into the tail vein (49.70 ± 6.08 RGCs/mm expressing BM88 at 7 days and 49.27 ± 2.23 RGCs/mm at 14 days; n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, in both cases the values were lower than the number of RGCs that expressed BM88 without injury (7 days: 11.0% less, 14 days: 20.2% less; n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). Although there was no statistical difference between the number of BM88 immunoreactive RGCs and Brn3a immunoreactive RGCs, it was statistically lower than the BM88 levels expected without injury. This suggests that the administration of HAPI cells into the tail vein resulted in the down regulation of BM88 from basal levels.

The down regulation of BM88 expression was more drastic following ONC injury. There were 43.2% fewer BM88 immunoreactive RGCs 4 days after HAPI cell were injected into the tail vein followed by ONC compared to the number of RGCs immunoreactive for Brn3a (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$), where there were only 24.18 ± 4.2 BM88 immunoreactive RGCs/mm (n=6; figure 6.7 and 6.9). This was also 50.0% lower than the number

of BM88 immunoreactive RGCs that would be expected 4 days after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). The number of BM88 immunoreactive RGCs 7 days after HAPI cell injection into the tail vein accompanied by an ONC was 53.0% lower than the density of Brn3a immunoreactive RGCs 7 days after HAPI cell were injected into the tail vein with ONC (10.83 ± 3.70 BM88 immunoreactive RGCs/mm; n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). However, this was not different from what would be expected after 7 days of ONC alone (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). There was no significant difference between the RGC densities of BM88 immunoreactive RGCs and Brn3a immunoreactive RGCs 14 days after HAPI cells were injected into the tail vein accompanied by an ONC (10.78 ± 4.95 BM88 immunoreactive RGCs/mm; n=6; figure 6.10; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, there were more BM88 immunoreactive RGCs 14 days after HAPI cells were injected into the tail vein with ONC than would be expected 14 days after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$).

6.1.5 Staining Intensity of BM88 after Injection of HAPI Cells into the Vitreous or Tail Vein

Regardless of how many BM88 immunoreactive RGCs remained after administration of HAPI cells into the vitreous or tail vein with or without crush, there was a decrease in the staining intensity of BM88 in RGCs in all conditions studied (figures 6.11-6.15). As soon as 4 days after HAPI cells were injected into the vitreous and tail vein, the BM88 intensity was only 61.55 ± 3.24% (n=6;

ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control and 64.13 ± 3.01% (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control, respectively. Then, 7 days after HAPI cells were injected into the vitreous or tail vein, the staining intensity was 46.53 ± 1.77% (HAPI IV; n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control and 45.47 ± 1.93% (HAPI TV; n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control. The staining intensity of BM88 14 days after HAPI cells were injected into the vitreous was 51.13 ± 1.90% (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control and 53.12 ± 1.86% (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control after they were injected into the tail vein.

The staining intensity was even less when there was an ONC after HAPI cells were injected into the vitreous or tail vein. The staining intensity of BM88 was 40.46 ± 2.83% (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) of the control after HAPI cells were injected into the vitreous accompanied by an ONC and 49.06 ± 2.09% of the control (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) after HAPI cells were injected into the tail vein with ONC. This reduction in staining intensity stayed consistent over the 14 day period studied, where the staining intensity was 29.61 ± 5.04% of the control (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) after intravitreal injection of HAPI cells accompanied by an ONC and 35.46 ± 2.09% of the control (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) after tail vein injection of HAPI cells with ONC. This suggests that after HAPI cells were injected into the tail vein or vitreous, there was a reduction

earlier and more severe than what was observed after ONC alone (figure 6.15). The intensity was lower after ONC, indicating that BM88 may be a sensitive indicator of the severity of the injury.

6.2 Discussion

Microglia are very sensitive to changes in their environment and respond to injury signals by migrating to the injury site and releasing many different soluble factors (Kreutzberg, 1996). Microglia can be either pro-inflammatory or anti-inflammatory depending on the type of injury they are responding to (Perry, Cunningham, & Boche, 2002). Neuronal-microglial signalling can also activate microglial cells. Extracellular adenosine 5'- triphosphates (ATPs) released by injured neurons acts as a chemoattractant and microglia migrate towards the injury mediated by $P2Y_{12}$ receptors (Haynes et al., 2006; Honda et al., 2001; Inoue, Koizumi, & Tsuda, 2007). ATP also acts on the microglial $P2X_4$ receptor causing the release of brain derived neurotrophic factor (BDNF). This can act on nearby neurons and alter the expression of anion transporters resulting in the change of GABA from being inhibitory to excitatory (Coull et al., 2005). This change may increase intracellular calcium levels and which leads to excitotoxicity (Arundine & Tymianski, 2003; Coull et al., 2005). ATP may also form part of an autocrine loop that increases calcium entry though activated P2X receptors (Khakh & North, 2006). ATP also binds to the P2Y receptors on neurons, which leads to IP_3 dependent release of calcium from internal stores (Masgrau et al., 2009). BM88 can reduce ATP evoked increases in intracellular calcium by acting

on the intracellular conversion of PLC on the P2Y receptors (Masgrau et al., 2009). BM88 is also associated with the endoplasmic reticulum, which is an important cellular calcium store (Masgrau et al., 2009). It is unclear what the role of BM88 is in the endoplasmic reticulum, but it may possibly play a role in preventing the release of calcium.

In chapter 5, we demonstrated that BM88 was down regulated 7 days after ONC. This may result in calcium dysregulation in the neuron leading to apoptosis. These studies showed that BM88 was down regulated earlier after a more severe injury or when there were HAPI cells located in the retina or optic nerve. Four days after HAPI cells were injected there was a decrease in the number of RGCs, as well as the staining intensity of BM88 of surviving RGCs that were still expressing BM88. The decrease in staining intensity of BM88 was lower in a more severe injury (after injection of HAPI cells and ONC vs. ONC alone or HAPI cells injected with no injury). The decrease in BM88 expression after HAPI cell injection into the vitreous or tail vein without ONC was interesting because it preceded any significant loss of RGCs, as measured by Brn3a, which was seen 14 days after HAPI cells were injected. This may be indicative of cellular dysfunction preceeding the destructive phases of apoptosis. The down regulation of BM88 may lead to death of the cells that are lost 14 days after injection.

The decrease in BM88 expression and loss of RGCs after HAPI cells were injected into the vitreous without ONC may be due to factors that were released by microglia and neuronal-microglial communication. Microglial activation is

regulated by internal levels of reactive oxygen species (ROS) (Block, Zecca, & Hong, 2007). There could be an increase in oxidative stress inside microglia due to the increase metabolic need of the cells inside the eye. Microglia produce pro-inflammatory ROS and cytokines, such as tumor necrosis factor α (TNFα), after activation. TNFα binds to a TNF-family of receptors on neurons and this increases production of intracellular ROS by mitochondria (Chandel, Schumacker, & Arch, 2001). Bcl-2 and Bcl-xL are anti-apoptotic proteins that prevent the damaging effects of the increase in mitochondrial ROS and maintain the mitochondria by keeping the redox status at control levels (Chandel et al., 2001). BM88 resembles Bcl-2 and has been shown to protect cells from apoptosis by inhibiting ERK activation of BAX (Georgopoulou et al., 2006; Masgrau et al., 2009). BM88 and Bcl-2 are both small transmembrane proteins, approximately 23 KD in size, that are found on mitochondria and the endoplasmic reticulum (Masgrau et al., 2009). Analysis shows that BM88 shares a 16 amino acid domain that resembles the death domain (BH3) on Bcl-2 (Masgrau et al., 2009). They are both functionally involved in neuronal differentiation, preventing apoptosis, and calcium mobilization (Lao & Chang, 2007; Masgrau et al., 2009). Therefore, like Bcl-2, BM88 is very important in maintaining the integrity of the mitochondria and preventing the cell from undergoing apoptosis. Evaluating BM88 expression may be useful in determining the health of the mitochondria and predicting the likelihood of apoptosis. The decrease in the number of RGCs that expressed BM88 at 4 (for HAPI TV) and 7 (for HAPI IV) days after injecting

HAPI cells without injury may have been predicting the death of RGCs seen at 14 days. In both cases, the number of cells that expressed BM88 and Brn3a were not statistically different after 14 days of injection, so most surviving RGCs expressed BM88 but it was at low levels, as indicated by the staining intensity. However, later in the time course, the staining intensity may have increased and future studies should explore this possibility.

One remaining question is how HAPI cells injected into the tail vein (without ONC) that never migrate to the optic nerve or retina are able to cause a decrease in the expression of BM88 in RGCs? Just as there is communication between neurons and microglia, there is communication between the immune system and the CNS. One hypothesis is that peripheral pro-inflammatory cytokines, particularly IL-1β, TNFα, and IL-6, communicate with the CNS to activate microglia and astrocytes to release cytokines locally (Teeling & Perry, 2009; Watkins & Maier, 2005). Peripheral cytokines cannot cross the blood brain barrier (BBB) but diffuse from the circulation in areas deficient in the BBB, such as the choroid plexus, or be transported into the CNS by endothelial cells (Banks, 2005). An alternative theory is that peripheral cytokine can activate BBB endothelial cells that release soluble factors activating nearby microglia and astrocytes (Turrin & Rivest, 2004). We suggest that communication through HAPI cells in the periphery led to BM88 down regulation in RGCs and also possibly activate resident microglia and astrocytes that may result in the loss of RGCs 14 days after injection. However, we predict that because the number of

RGCs that were immunoreactive for BM88 was not significantly different from those expressing Brn3a 14 days after HAPI cells were injected, retinas examined at later time points might have a rebound in the BM88 expression by RGCs.

BM88 is also involved in neuronal cell cycle exit and differentiation (Georgopoulou et al., 2006). The down regulation of BM88 after injury or neuroinflammation may suggest that neurons may try to re-enter the cell cycle, even though they are post mitotic. This has been shown in neurodegenerative disorders such as Alzheimer's disease, ALS, and Parkinson's disease (Arendt, 2002; Höglinger et al., 2007; Nguyen et al., 2003). The "two hit hypothesis" (suggested for Alzheimer's disease but may apply to other neurodegenerative disorders as well) states that both mitogenic dysregulation and oxidative stress are required and sufficient to cause neuronal cell death (Zhu, Lee, Perry, & Smith, 2007; Zhu, Raina, Perry, & Smith, 2004). The high amounts of oxidative stress required to cause apoptosis of neurons is often not seen in neurodegenerative diseases, however, low concentrations of oxidants can induce an adaptive response by neurons (Wiese, Pacifici, & Davies, 1995; Zhu et al., 2007). The cells that re-enter the cell cycle may be more susceptible to damage by oxidative stress (Zhu et al., 2007). Therefore, the oxidative stress produced by the addition of HAPI cells resulted in the down regulation of BM88 making the RGCs more vulnerable to death. The activation of HAPI cells by ONC may result in the additional death of RGCs because the down regulation of BM88 following ONC

injury has made them more susceptible to apoptosis from oxidative stress (ROS) and cytokines produced by activated HAPI cells.

Down regulation of BM88 may be a good indicator of abnormal RGC functioning. BM88 down regulation preceded the death of the RGC and its expression, as measured by the number of RGCs that expressed the protein and the staining intensity of those cells, was lower when the injury was more severe. The number of RGCs that expressed BM88 was lower when HAPI cells were injected into the vitreous or tail vein accompanied by an ONC injury than the number of RGCs that survived after ONC. The staining intensity of BM88 after HAPI cells were injected into the vitreous or tail vein with an ONC was lower than the staining intensity after the HAPI cells were injected without injury and the staining intensity after ONC alone. This down regulation of BM88 may be due to communication between the neuron and microglia and communication between the immune system and CNS. Oxidative stress and cytokines produced by HAPI cells may result in neuronal stress resulting in them down regulating BM88. This down regulation may indicate impaired calcium regulation, initiation of apoptosis and mitochondrial dysfunction, and/or cell cycle dysregulation. Furthermore an increase in intracellular calcium levels would lead to cell death through apoptosis. Inhibition of apoptosis by BM88 can be released resulting in the death of the cell. Finally, the loss of the inhibition of cyclin D, which prevents cell cycle entry, can result in cell cycle re-entry, making the neuron more vulnerable to oxidative stress. Activated microglia produced cytokines and oxidative stress that resulted

in the increased loss of RGCs than what was observed after ONC alone. In that regard, BM88 expression is neuroprotective and understanding the mechanisms by which BM88 exerts its influence may result in the discovery of new therapeutic targets for CNS injury, including glaucoma. BM88 expression in neurons may also be a good reporter for neuronal health and functioning.

6.3 Figures and Tables

Figure 6.1: Co-localization of RGCs labelled with Brn3a with BM88 4-14 days after injection of HAPI microglial cells into the vitreous with no injury. In control retinas, most Brn3a immunoreactive RGCs (green) co-localized with BM88 (red). However, there was evidence that after HAPI cell injection into the vitreous, even without injury, there was loss of BM88 expression by RGCs. This loss preceded the death of the cell that was evident by the Brn3a staining (dotted circles around the cells).

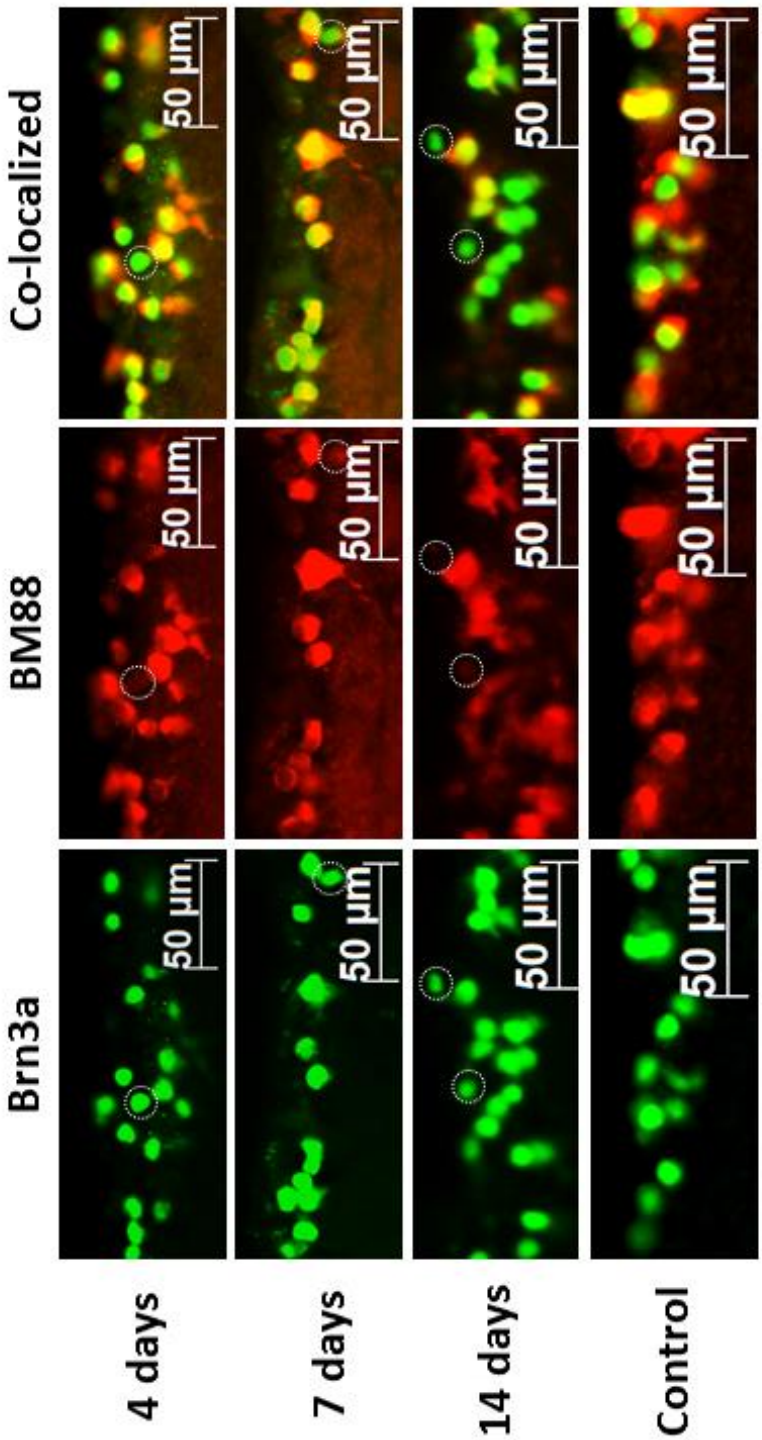

Co-localized
BM88
Brn3a
4 days
7 days
14 days
Control
50 µm

Figure 6.2: Co-localization of RGCs labelled with Brn3a with BM88 4-14 days after injection of HAPI microglial cells into the vitreous and optic nerve crush. Most Brn3a immunoreactive RGCs (green) co-localized with BM88 (red) in control retinas with no injury. After HAPI cells were injected into the vitreous and there was an ONC injury, there were many RGCs that did not express BM88 (dotted circles). Therefore, BM88 was down regulated before the death of the RGC.

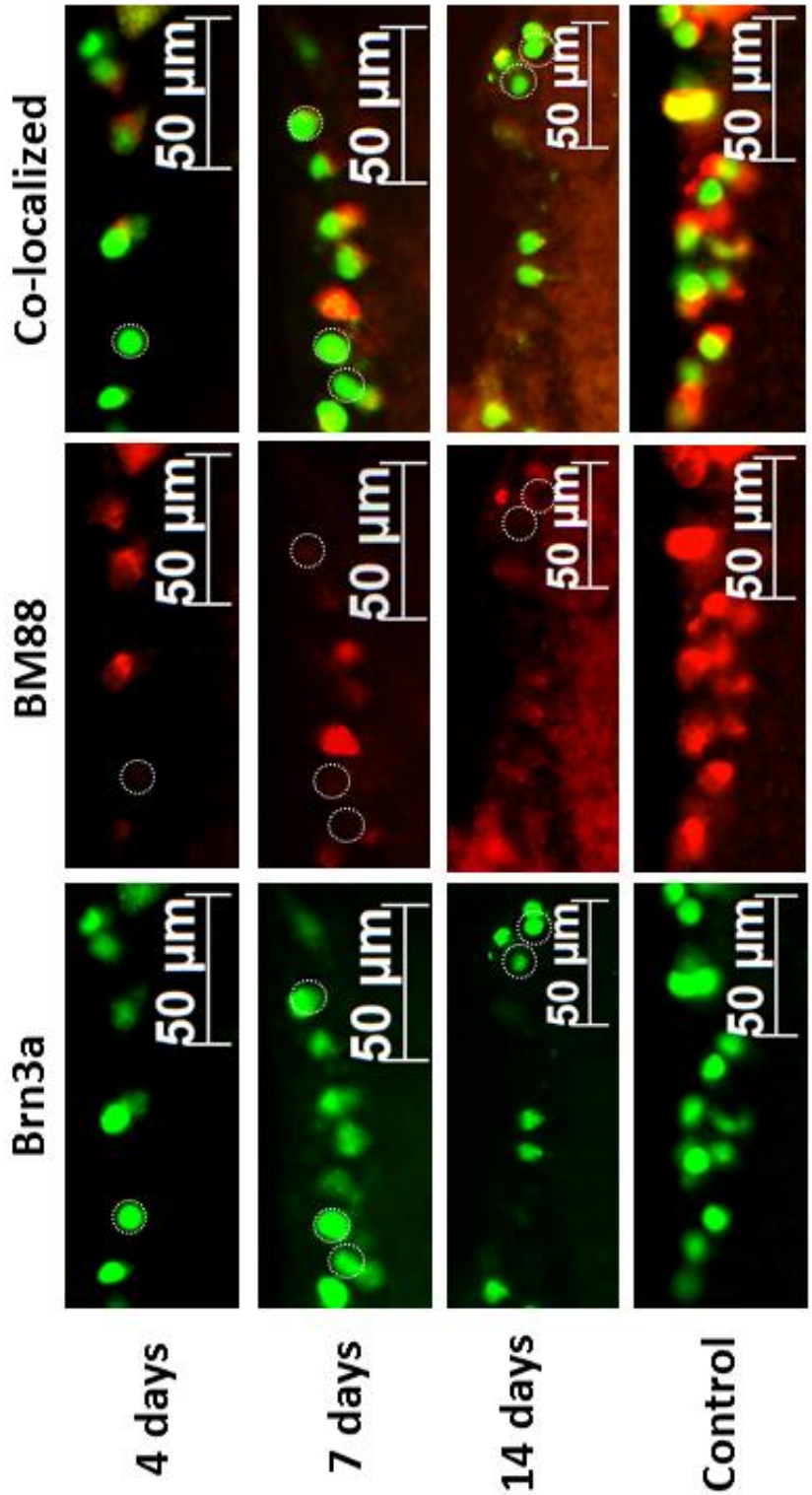

Brn3a
BM88
Co-localized
4 days
7 days
14 days
Control
50 µm

Figure 6.3: The loss of RGCs after injection of HAPI cells into the vitreous. There was no RGC loss after HAPI cells were injected into the vitreous in the absence of injury (HAPI IV; green circle) until 14 days after injection. The decline in RGC density 14 days after injection of HAPI cells into the vitreous was significantly different from the control (grey dotted line). There was a significant loss of RGCs greater than what would be expected after ONC alone (grey line) 4 and 7 days after HAPI cells were injected into the vitreous followed by an ONC (red square).

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

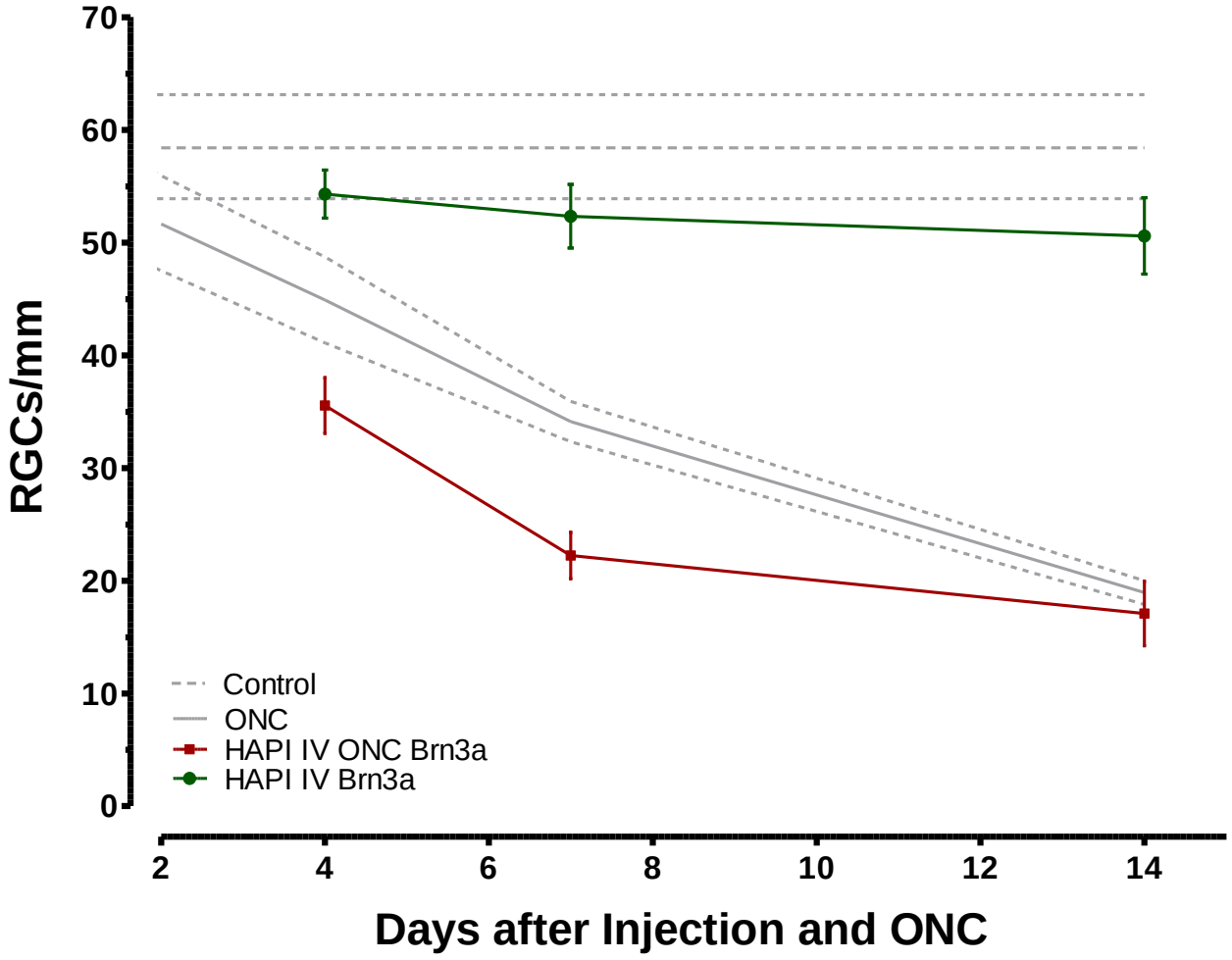

70
60
50
40
30
20
10
0
RGCs/mm
Control
ONC
HAPI IV ONC Brn3a
HAPI IV Brn3a
2
4
6
8
10
12
14
Days after Injection and ONC

Figure 6.4: The number of RGCs expressing BM88 after HAPI cells were injected into the vitreous. After ONC injury (grey line) there was a loss of RGCs that expressed BM88 compared to the number of RGCs that expressed BM88 without any injury (grey dotted line). When HAPI cells were injected into the vitreous without any injury (HAPI IV; red circle), there was a decrease in the number of RGCs that expressed BM88 after 4 days and it was maintained over the 14 day period. There was a more severe decline in RGCs that expressed BM88 4 days after HAPI cells were injected into the vitreous with an ONC (HAPI IV ONC; blue triangle) compared to the control and this loss is maintained over the 14 day period. However, this decline was only significantly lower 4 days after injection when compared to the loss expected after ONC alone. This suggests that after a more severe injury (in this case, HAPI cells injected into the vitreous), there is an earlier and more severe decline in RGCs that expressed BM88. However, these declines in RGCs that expressed BM88 preceded the death of the RGC.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

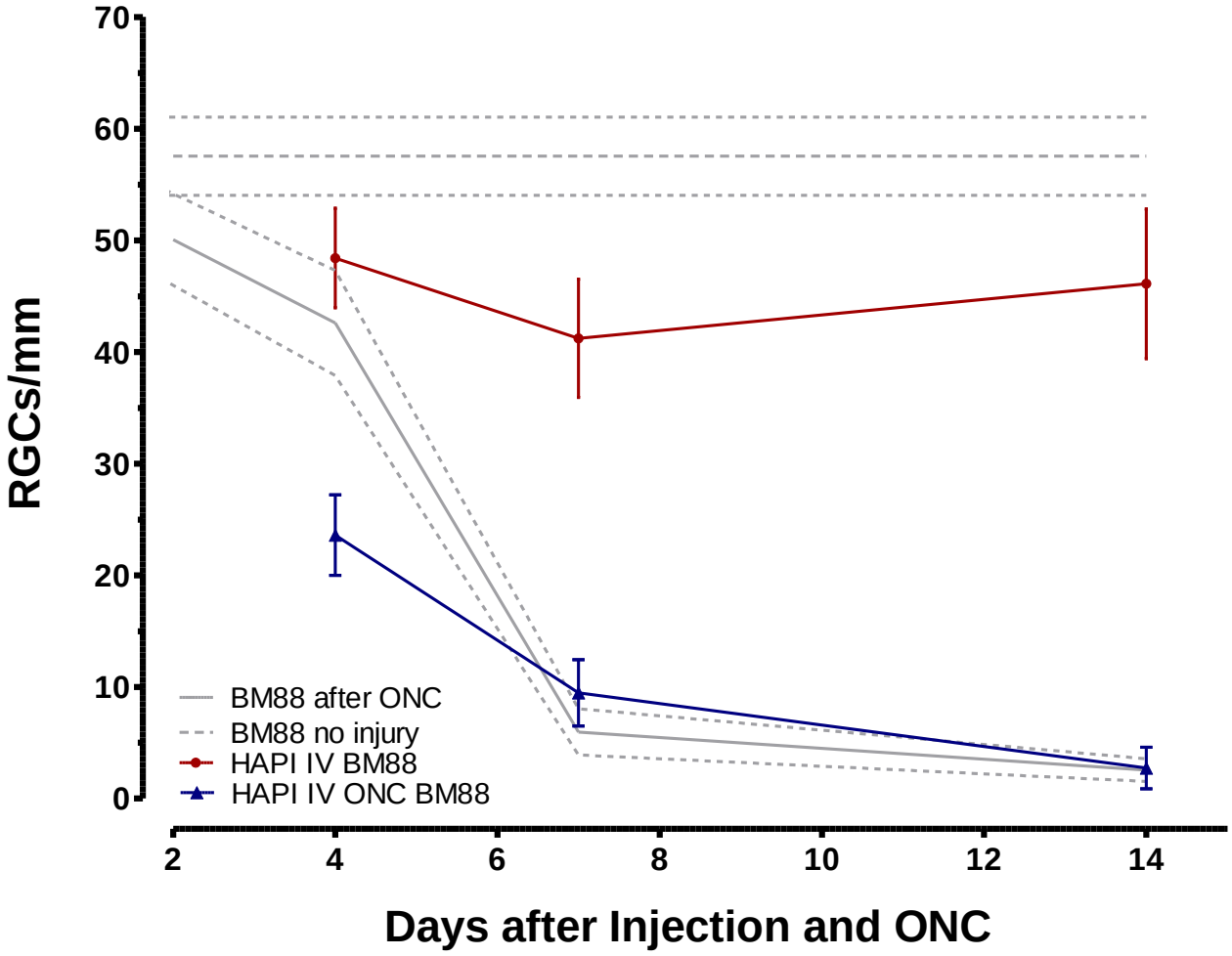
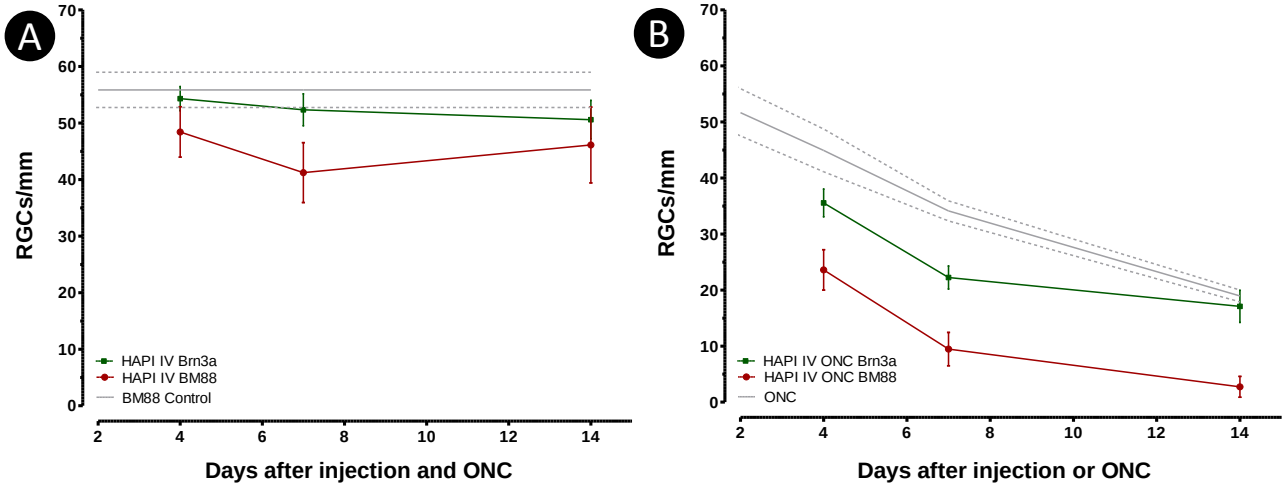

RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after Injection and ONC
BM88 after ONC
BM88 no injury
HAPI IV BM88
HAPI IV ONC BM88

Figure 6.5: The RGC densities of cells that express Brn3a and BM88 after injection of HAPI cells into the vitreous over a 14 day period. The decline in RGCs that expressed BM88 preceded the death of the RGCs as it is evident by the presence of the Brn3a positive cells. **(A)** There was no loss of Brn3a immunoreactive RGCs (except at 14 days) after HAPI cells are injected into the vitreous (green square) when compared to the control. There was a decline in the BM88 immunoreactive RGCs after HAPI cell injection (red circles) compared to the number of BM88 immunoreactive RGCs after ONC alone. However, when compared to the RGC density of RGCs that expressed Brn3a, there was only a decline in BM88 expressing cells at 7 days after injection. **(B)** There was a pronounced decrease in BM88 expressing RGCs after HAPI cells are injected into the vitreous with an injury to the optic nerve (red circles). This loss represented a down regulation in BM88 expression by RGCs because there was a greater number of RGCs that expressed Brn3a. This suggested that BM88 may be neuroprotective since it was down regulated before death of the cell. **(C)** The severity of the injury determined when and how much BM88 was down regulated. There were more BM88 expressing RGCs 4 days after ONC alone (pink dotted line) than 4 days after HAPI cells were injected into the vitreous with ONC (red squares).

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


A
RGCs/mm
HAPI IV Brn3a
HAPI IV BM88
BM88 Control
Days after injection and ONC
B
RGCs/mm
HAPI IV ONC Brn3a
HAPI IV ONC BM88
ONC
Days after injection or ONC

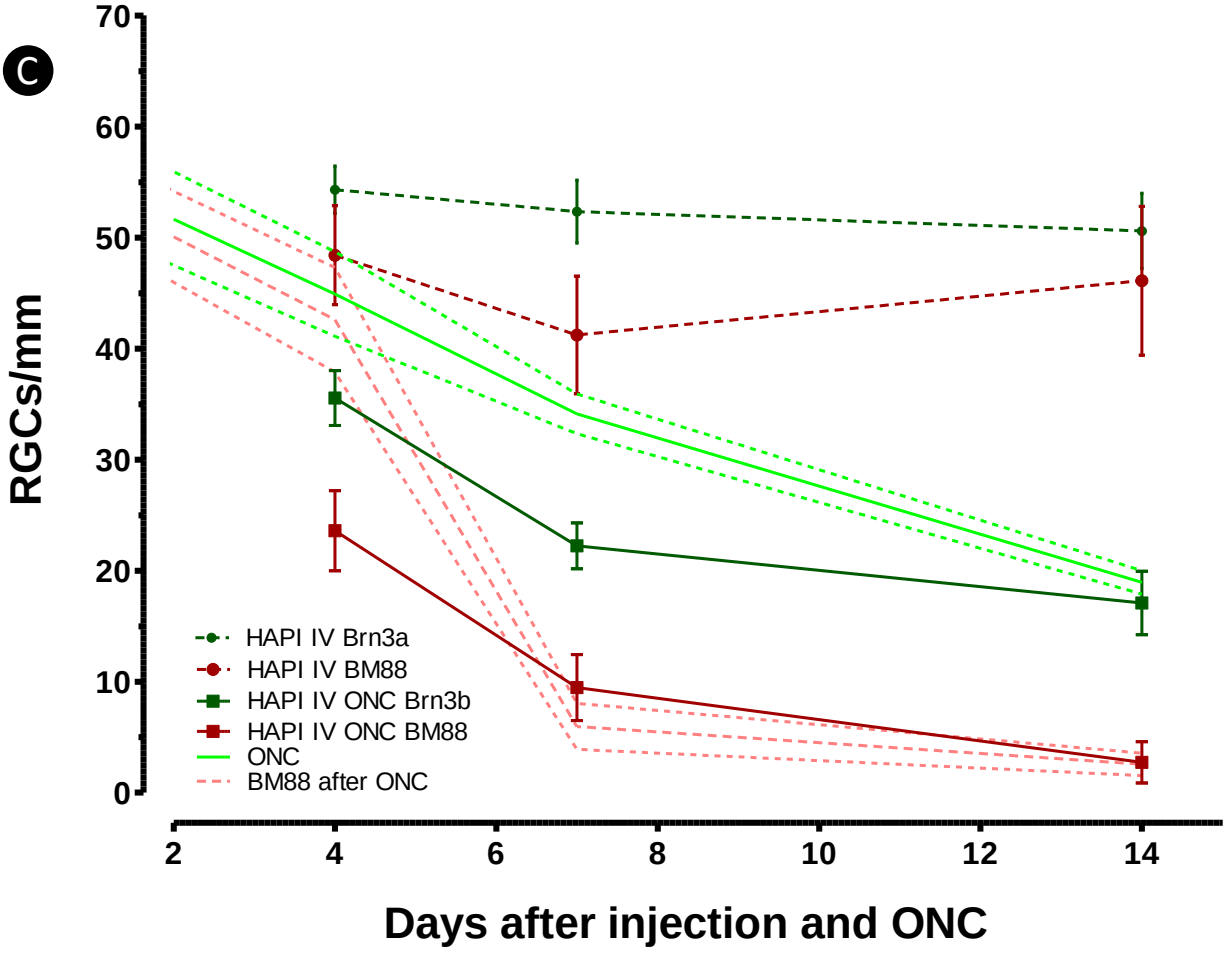

C
RGCs/mm
HAPI IV Brn3a
HAPI IV BM88
HAPI IV ONC Brn3b
HAPI IV ONC BM88
ONC
BM88 after ONC
Days after injection and ONC

Figure 6.6: Co-localization of RGCs labelled with Brn3a with BM88 4-14 days after injection of HAPI microglial cells into the tail vein with no injury. In control retinas, most Brn3a immunoreactive RGCs (green) co-localized with BM88 (red). After HAPI cell were injected without injury to the optic nerve, there was a loss of BM88 expression by RGCs. This loss preceded the death of the cell, which is evident by the Brn3a staining (dotted circles around the cells).

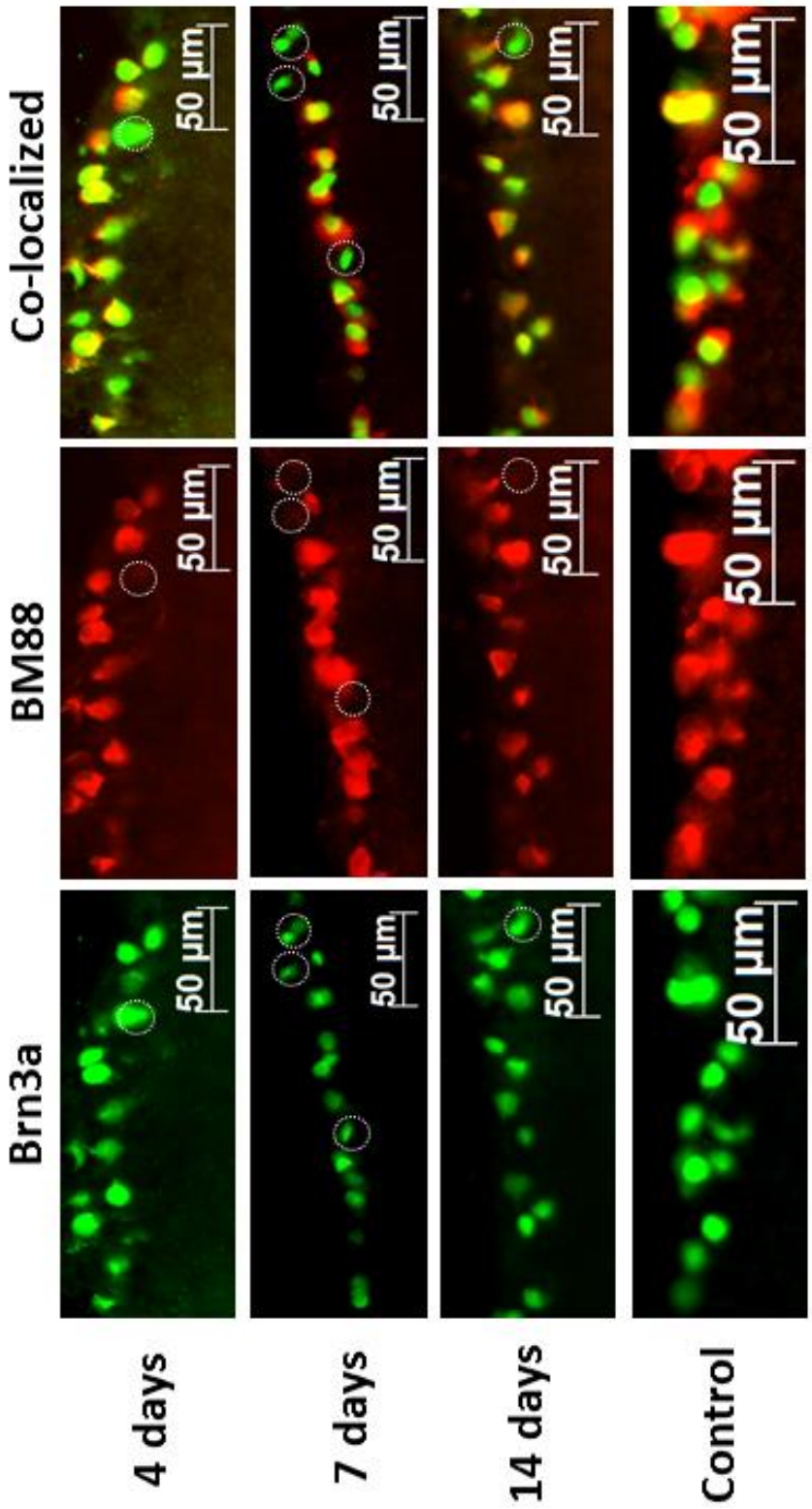

Brn3a
BM88
Co-localized
4 days
7 days
14 days
Control
50 µm

Figure 6.7: Co-localization of RGCs labelled with Brn3a with BM88 4-14 days after injection of HAPI microglial cells into the tail vein and optic nerve crush. Most Brn3a immunoreactive RGCs (green) co-localized with BM88 (red) in control retinas with no injury. After HAPI cells were injected and after ONC injury, there were many RGCs that did not express BM88 (dotted circles). Therefore, like after intravitreal injection and ONC, BM88 may be down regulated before the death of the RGC.

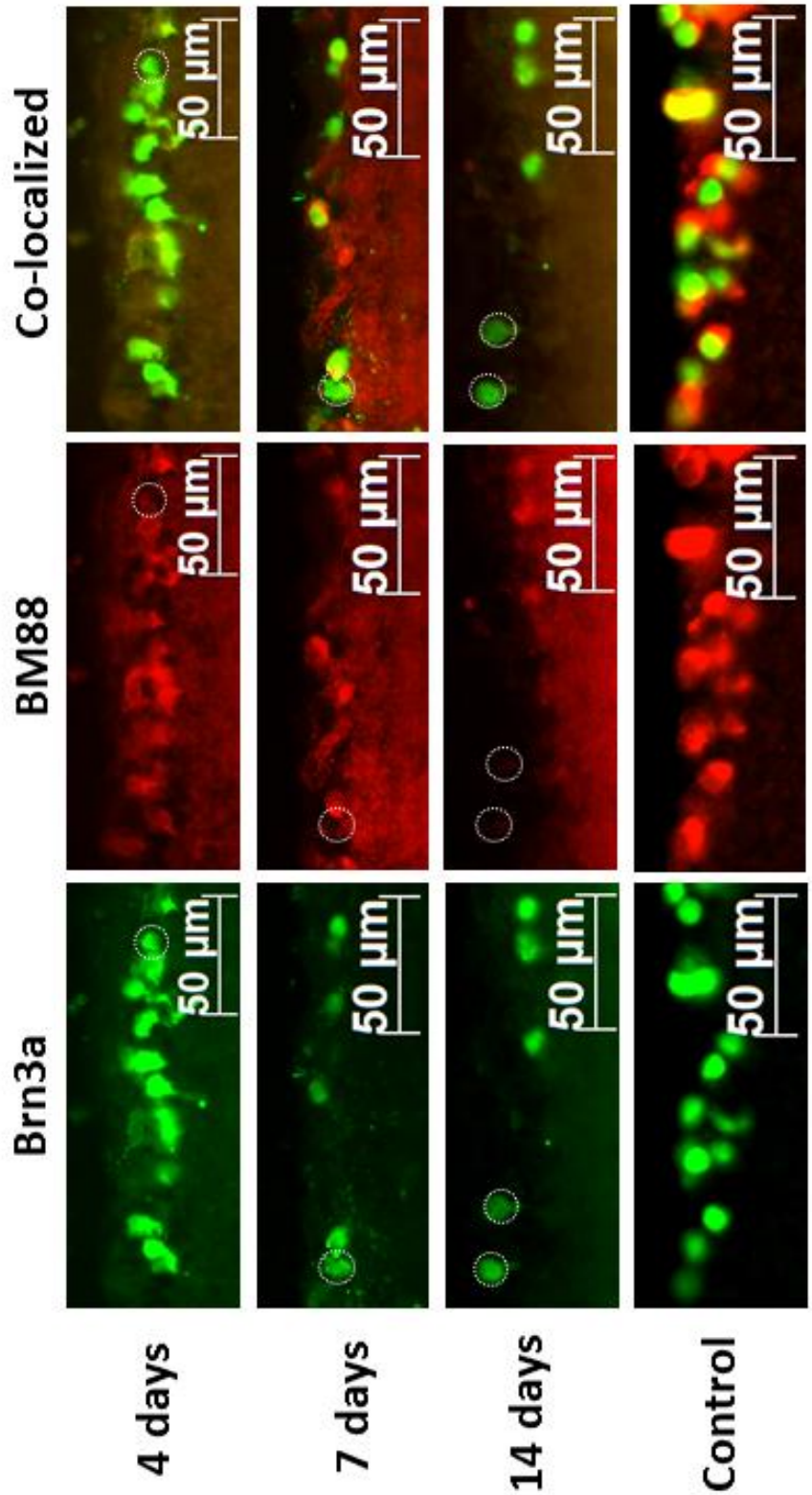

Brn3a
BM88
Co-localized
4 days
7 days
14 days
Control
50 µm

Figure 6.8: The loss of RGCs after injection of HAPI cells into the tail vein. There was no RGC loss after HAPI cells were injected into the vitreous in the absence of injury (HAPI TV; green circle) until 14 days after injection. The loss of RGCs 14 days after injection was statistically different from the control (grey dotted line). There was a loss of RGCs greater than what would be expected after ONC alone (grey line) 7 days after HAPI cells were injected followed by an ONC (red square). However, the RGCs survival went back to expected values after 14 days of injury and injection.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

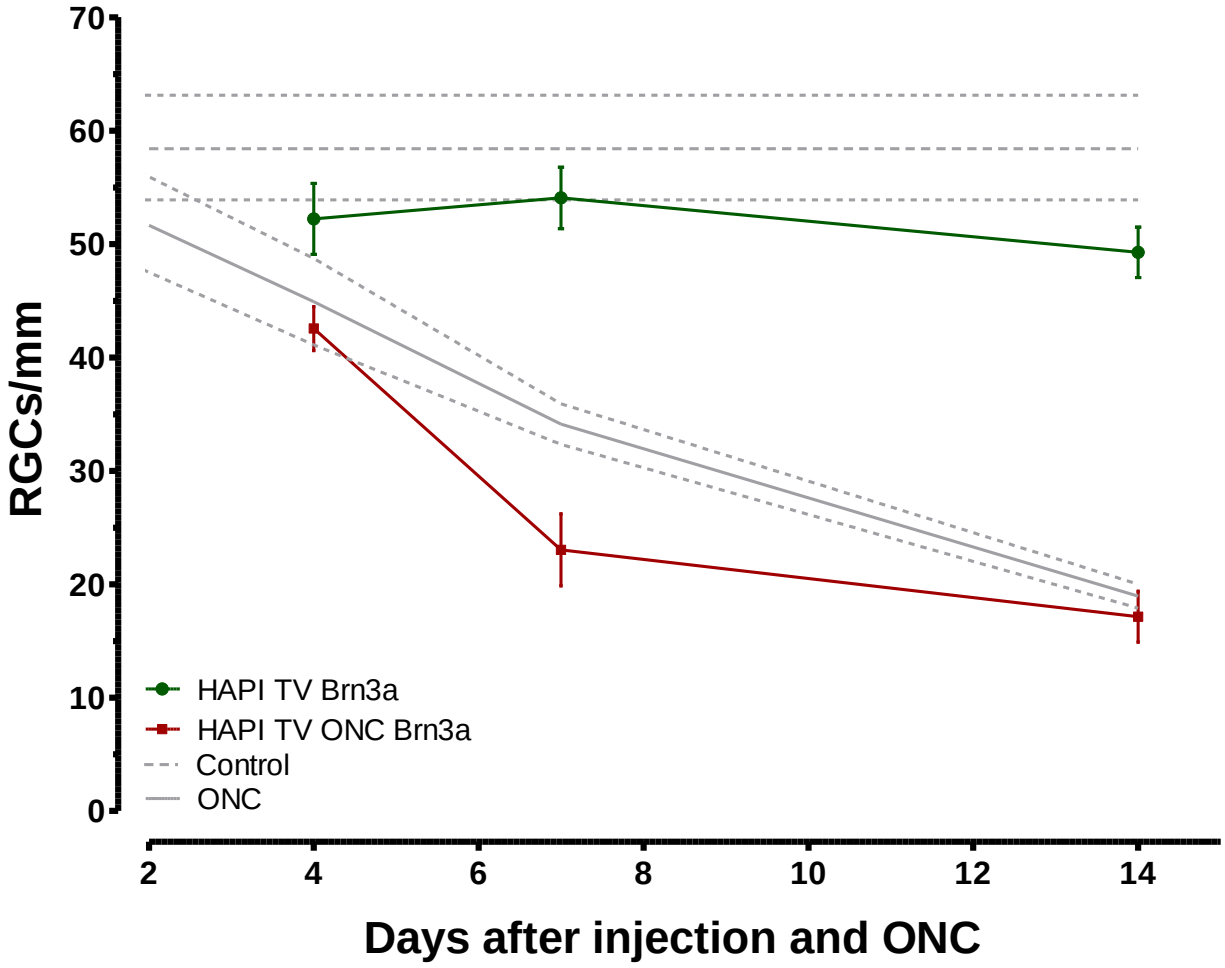

70
60
50
40
30
20
10
0
RGCs/mm
HAPI TV Brn3a
HAPI TV ONC Brn3a
Control
ONC
2
4
6
8
10
12
14
Days after injection and ONC

Figure 6.9: The number of RGCs expressing BM88 after HAPI cells were injected into the tail vein. After ONC injury (grey line) there was a loss of RGCs that expressed BM88 compared to the number of RGCs that expressed BM88 without any injury (grey dotted line). There was a decrease in the number of RGCs that expressed BM88 after HAPI cells are injected into the tail vein without injury (HAPI TV; red circles), as soon as 4 days after injection and it is sustained over the 14 day period. This decrease was similar to what is seen 4 days of ONC. There was also a decline in RGCs expressing BM88 4 days after HAPI cells were injected into the tail vein with ONC (HAPI TV ONC; blue triangle) that was sustained over the 14 day period. The loss was greater than what was expected 4 days after ONC alone but there were more RGCs contained BM88 14 days after injection and ONC.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

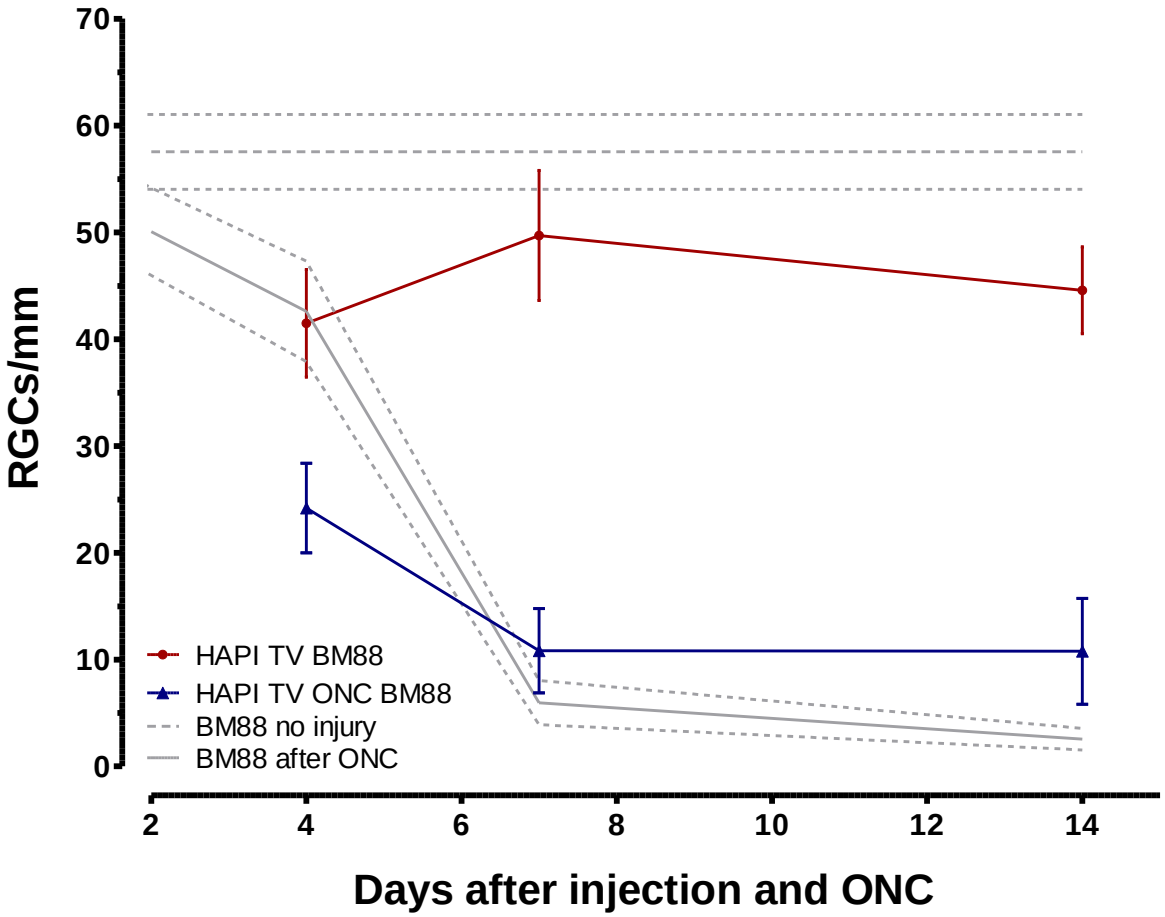
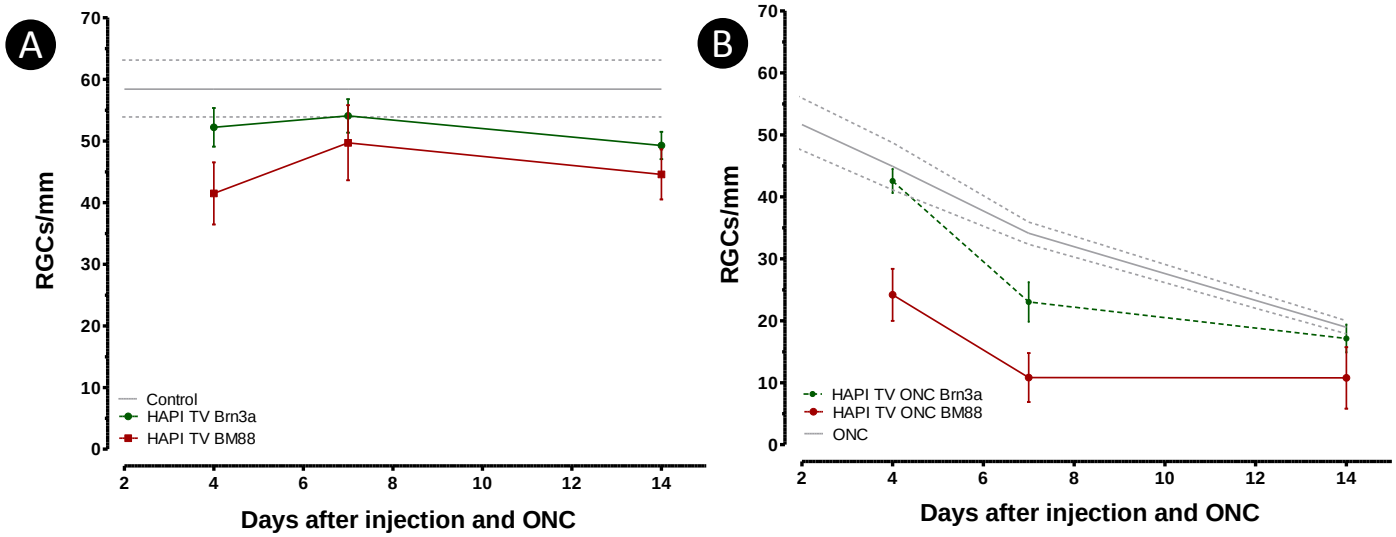

70
60
50
40
30
20
10
0
RGCs/mm
HAPI TV BM88
HAPI TV ONC BM88
BM88 no injury
BM88 after ONC
2
4
6
8
10
12
14
Days after injection and ONC

Figure 6.10: The RGC densities of cells that express Brn3a and BM88 after injection of HAPI cells into the tail vein over a 14 day period. (A) There was a statistically lower amount of RGCs immunoreactive for BM88 (red squares) than immunoreactive for Brn3a (green circles). However, 7 and 14 days after HAPI cell were injected into the tail vein without injury, the number of RGCs immunoreactive for Brn3a and BM88 were statistically not different. **(B)** The number of RGCs that expressed BM88 (red) were lower than the ones expressing Brn3a (green) after tail vein injection of HAPI cells with ONC over the 14 day period. This suggests that BM88 expression can indicate the health of the RGC and is sensitive to a pro-inflammatory environment. **(C)** RGCs down regulated BM88 before the death of the RGC. The more severe the injury, the less BM88 expressing RGCs there were.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


A
RGCs/mm
Control
HAPI TV Brn3a
HAPI TV BM88
Days after injection and ONC
B
RGCs/mm
HAPI TV ONC Brn3a
HAPI TV ONC BM88
ONC
Days after injection and ONC

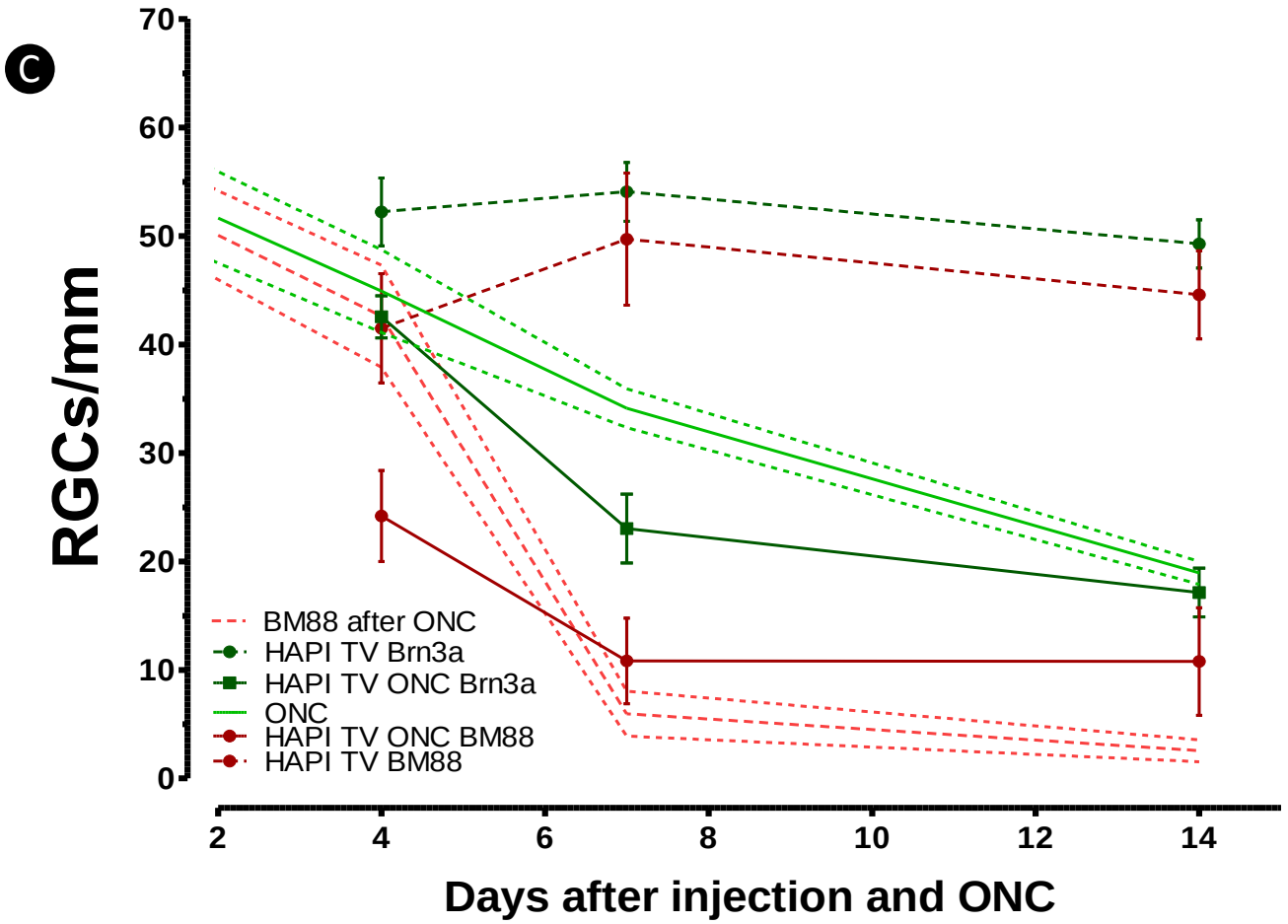

C
RGCs/mm
BM88 after ONC
HAPI TV Brn3a
HAPI TV ONC Brn3a
ONC
HAPI TV ONC BM88
HAPI TV BM88
Days after injection and ONC

Figure 6.11: Down regulation of BM88 after HAPI cells are injected into the vitreous with or without ONC injury. The images were taken using standardized camera settings with the same exposure. In control retinas, there was bright labelling of RGCs with BM88. However, the staining becomes dimmer 4 days after injection and the loss was sustained over the 14 day period. The BM88 down regulation after HAPI cell injection and ONC (HAPI IV ONC) was visibly greater than when there is no injury.

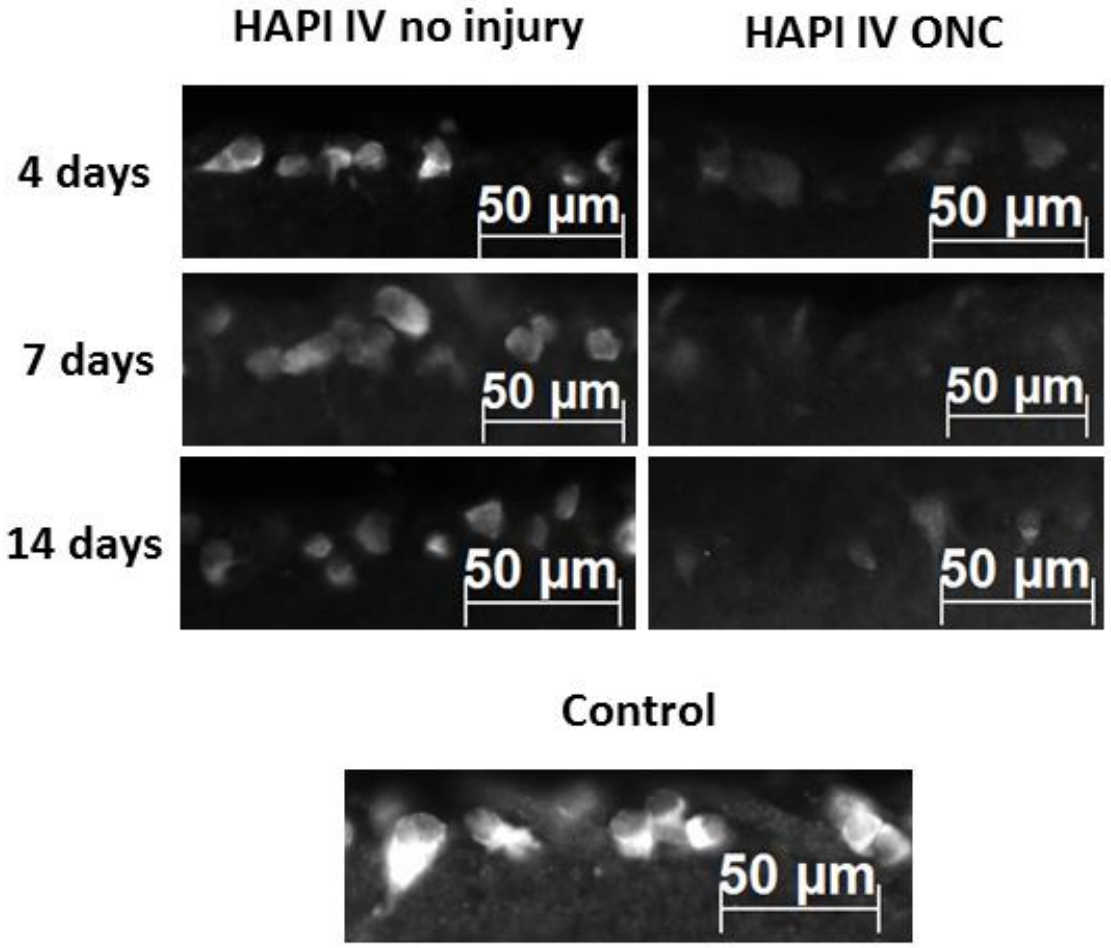

HAPI IV no injury
HAPI IV ONC
4 days
7 days
14 days
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm
Control
50 µm

Figure 6.12: Staining intensity of BM88 after injection of HAPI cell into the vitreous with and without ONC over a 14 day period. Four days after ONC, the staining intensity increased from control levels. The staining intensity decreased by half 7 days after ONC (black dotted line). However, 4 days after injecting HAPI cells into the vitreous there was a decrease in the staining intensity of BM88 by surviving RGCs and the staining intensity stayed low in surviving RGCs over the 14 day period. The decrease in staining intensity was lower after HAPI cells were injected and there was an ONC injury (red squares). BM88 down regulation may be an indicator of abnormal RGC functioning and be sensitive to neuroinflammation.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

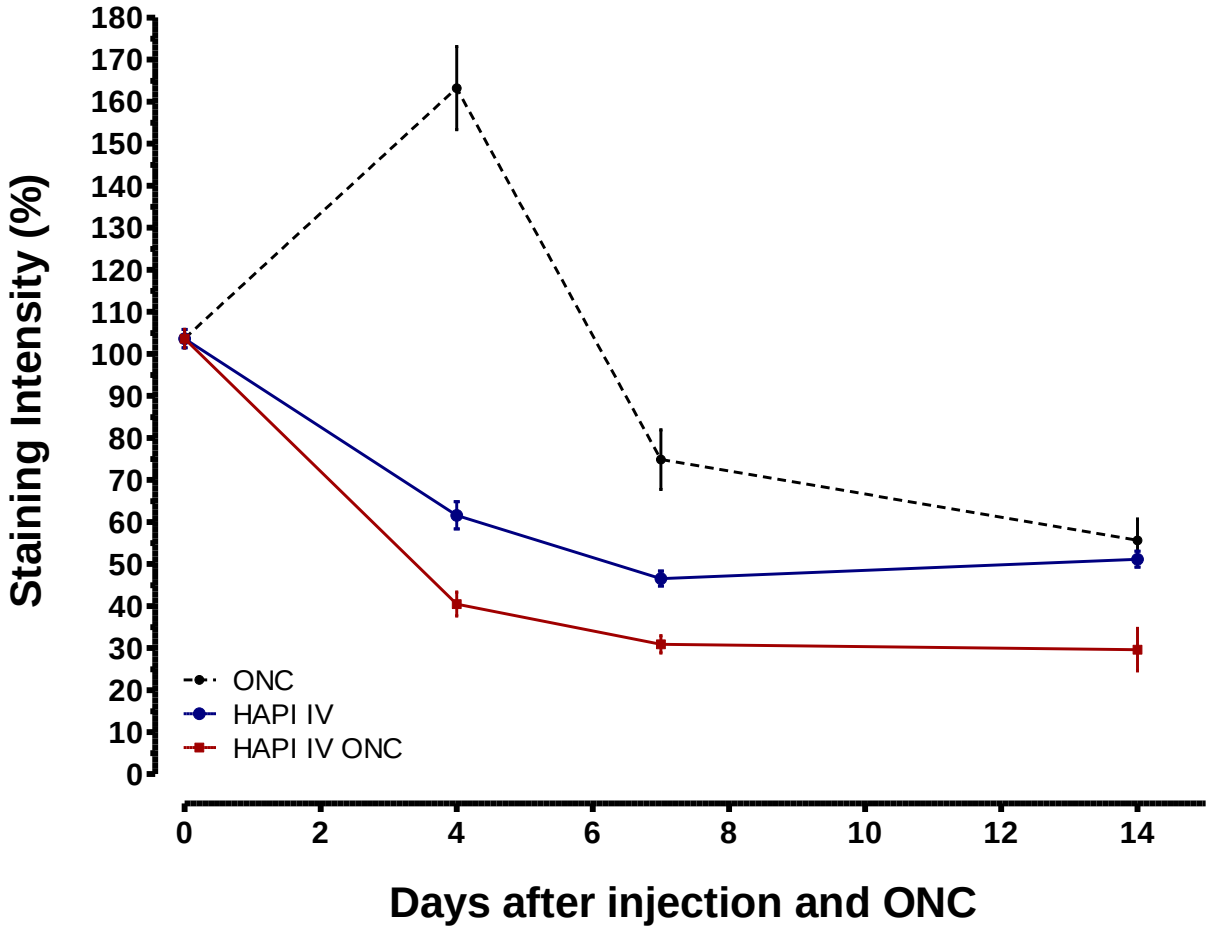
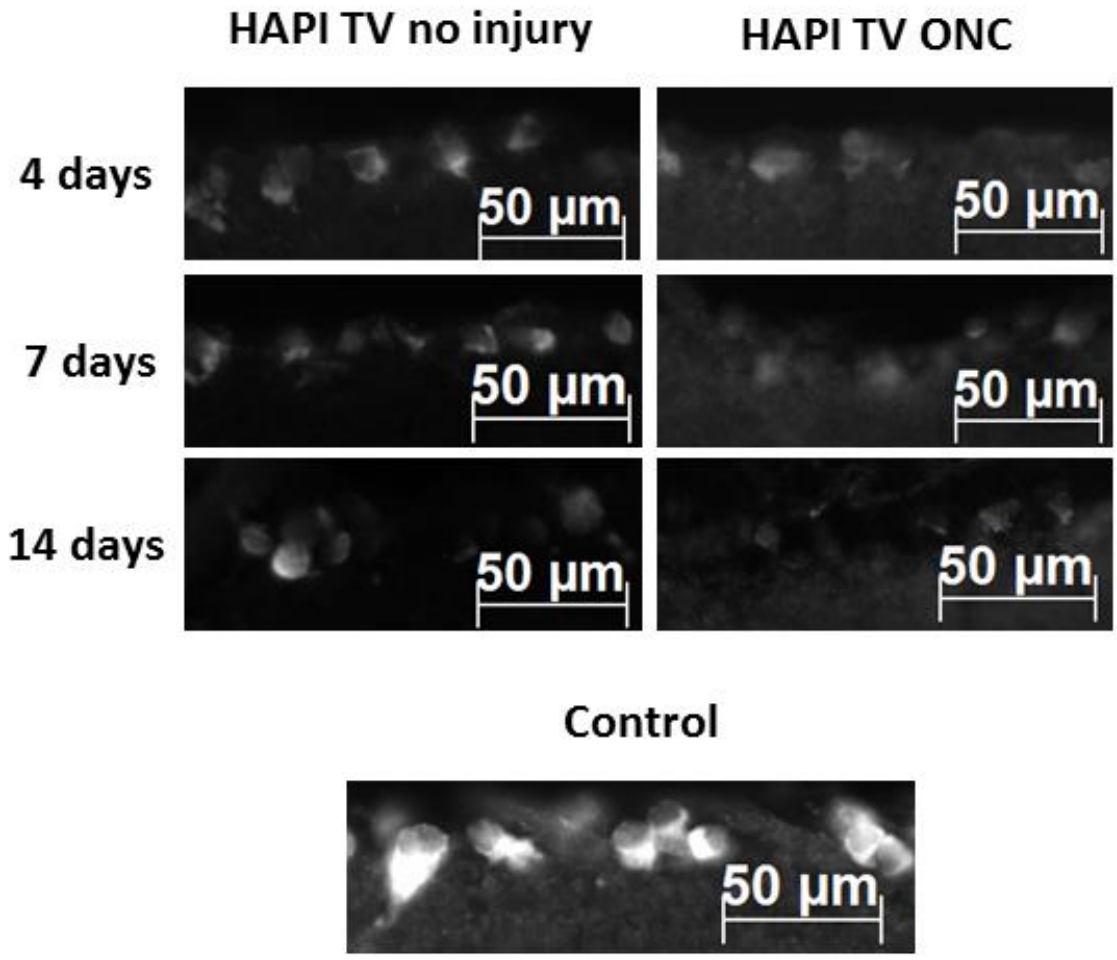

Staining Intensity (%)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ONC
HAPI IV
HAPI IV ONC
0
2
4
6
8
10
12
14
Days after injection and ONC

Figure 6.13: Down regulation of BM88 after HAPI cells are injected into the tail vein with or without ONC injury. The images were taken using standardized camera settings with the same exposure. In control retinas, there was bright labelling of RGCs with BM88. The staining intensity decreased 4 days after injection and the loss is sustained over the 14 day period. The BM88 down regulation after HAPI cell injection and ONC (HAPI TV ONC) was visibly greater than when there was no injury.


HAPI TV no injury
HAPI TV ONC
4 days
7 days
14 days
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm

Control
50 µm

Figure 6.14: Staining intensity of BM88 after injection of HAPI cell into the tail vein with and without ONC over a 14 day period. Four days after ONC, the staining intensity increased from control levels. The staining intensity decreased by half 7 days after ONC (black dotted line). It was 4 days after injecting HAPI cells into the tail vein that there was a decrease in the staining intensity of BM88 by surviving RGCs. The decrease in staining intensity was lower for after HAPI cells were injected and there was an ONC injury (red squares). The loss was maintained over the 14 day period. This loss was greater than the loss seen after ONC at 4 and 7 days but it went back to the levels seen 14 days after ONC when HAPI cells were injected into the tail vein for 14 days. The decrease in staining intensity occured before the death of the RGC itself.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

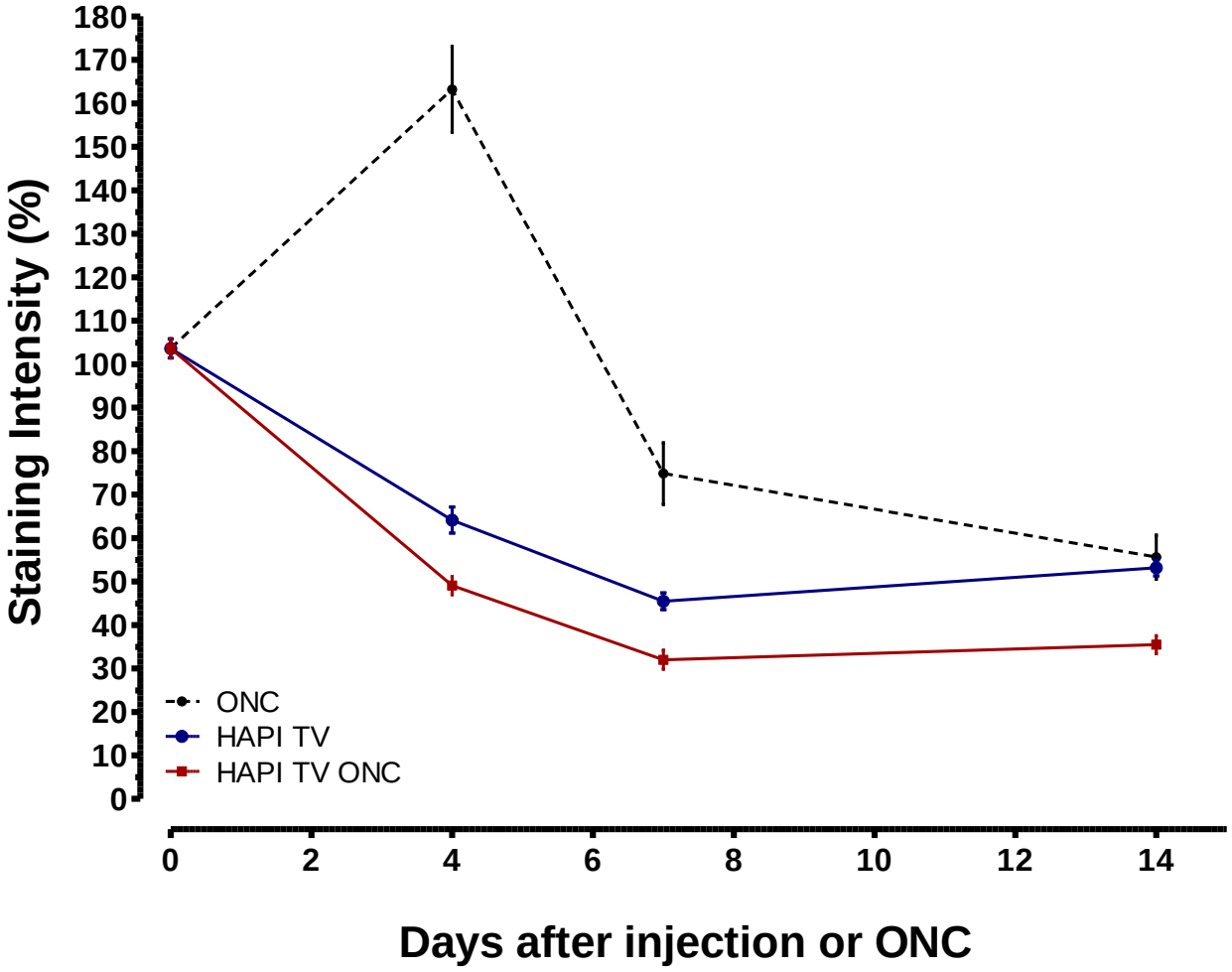
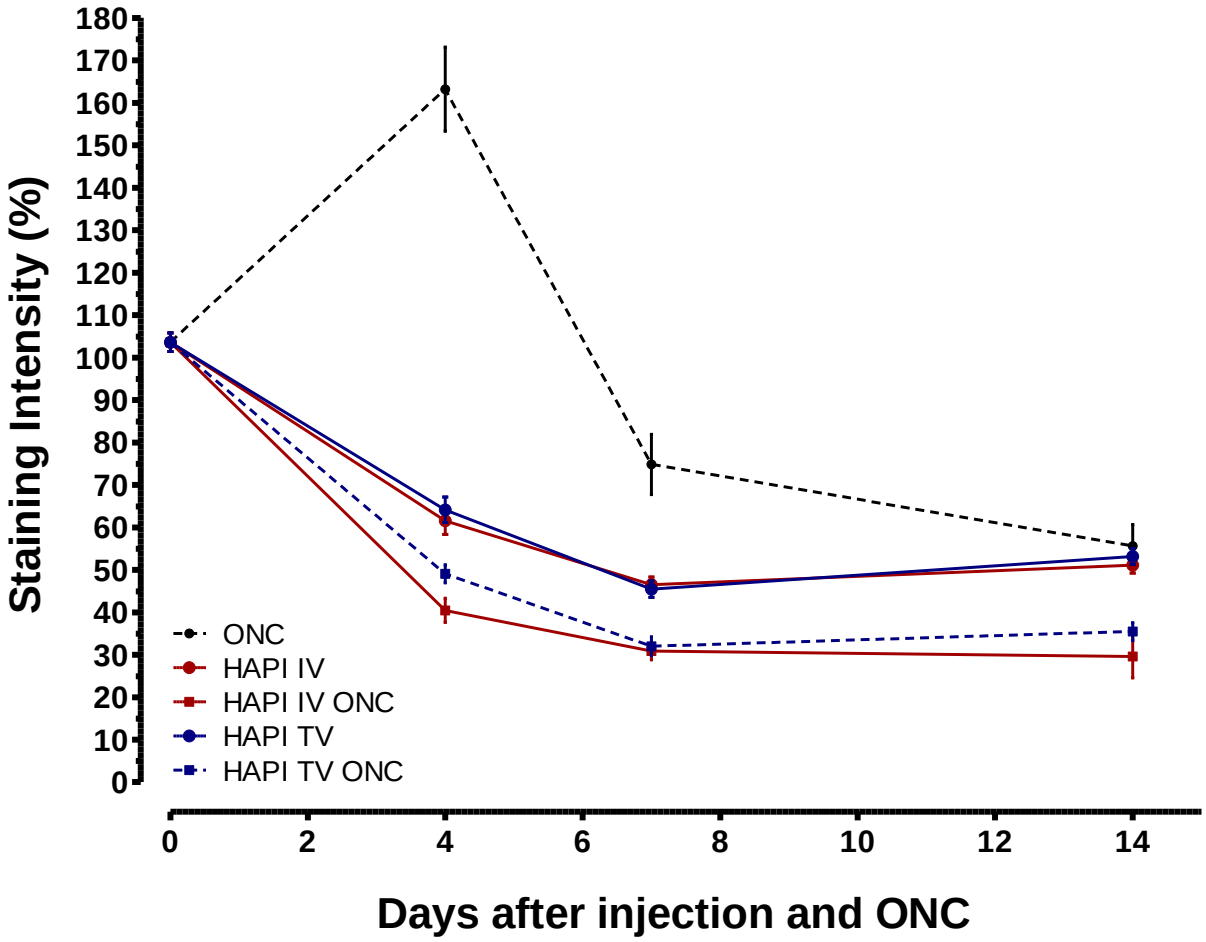

Staining Intensity (%)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ONC
HAPI TV
HAPI TV ONC
0
2
4
6
8
10
12
14
Days after injection or ONC

Figure 6.15: Staining intensity of BM88 after injection of HAPI cell into the vitreous or tail vein with and without ONC over a 14 day period. The decrease in staining intensity of BM88 was the same regardless of the injection route but dependant on the type of injury. The staining intensity was the lowest when HAPI cells were injected with an ONC (squares). There was a greater decrease in BM88 staining intensity when HAPI cells were injected into the rat without injury than when there was an ONC without any injection (black dotted line).

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


Staining Intensity (%)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ONC
HAPI IV
HAPI IV ONC
HAPI TV
HAPI TV ONC
0
2
4
6
8
10
12
14
Days after injection and ONC

CHAPTER 7: RETINAL GANGLION CELL SURVIVAL AFTER INJECTION OF LPS ACTIVATED HAPI CELLS

7.1 Results

The purpose of this study was to activate microglial HAPI cells *in vitro* and inject them into an animal to study the effect of LPS activation of microglia on injured RGCs without the activation of other cell types. It was my hypothesis that hyper-activating HAPI (highly aggressive proliferation immortalized) microglial cells with LPS would lead to a pro-inflammatory response resulting in increased death of RGCs. An overview of this chapter is in Appendix IV.

7.1.1 Co-localization of Brn3b with Fluorogold Labelled RGCs

Over 90% of rat RGCs are thought to express Brn3b and it has been used as a reliable marker for RGCs (Nadal-Nicolás et al., 2009). In control retinas, (figure 7.1 G-I), 94% of Brn3b immunoreactive cells co-localized with fluorogold (FG) labelled RGCs. The high degree of co-localization remained constant over 7 days following ONC (figure 7.1 A-C), where 92% of FG labelled RGCs were immunoreactive for Brn3b. After 14 days following ONC, 80% of FG labelled RGCs were immunoreactive for Brn3b. There were some FG labelled cells that were not immunoreactive for Brn3b (figure 7.1 arrow heads), particularly after ONC. These cells were most likely microglial cells rather than RGCs that had phagocytosed FG and became transcellularly labelled. This would mean that the numbers obtained by Brn3b counts may be more reliable than FG due to the lower likelihood of identifying false positive cells. However, in some cases, there were some cells that were immunoreactive for Brn3b but contained no FG (dotted circles). These cells may have been amacrine cells but the intensity of staining

was only slightly higher than background staining. Brn3b therefore proved to be a reliable marker for RGCs so it was used for subsequent studies where the retina was treated with LPS activated HAPI cells.

7.1.2 Endotoxin Levels in HAPI Cell Solution

An endotoxin standard between 0 and 0.1 EU/mL was used to determine the levels of endotoxins in the injection solution of LPS activated HAPI cells and non-activated HAPI cells. Both HAPI cells and LPS activated HAPI cells had similar absorbance readings at 545 nm of 0.1357 and 0.0817, respectively (figure 7.2). This equates to an endotoxin concentration of 0.104 EU/mL and 0.095 EU/mL. Values lower than 0.5 EU/mL are considered non-pyrogenic (Ding & Ho, 2001). Both solutions contained 5 times less endotoxins per millilitre than what is the amount considered to be pyrogenic.

7.1.3 Migration of HAPI Cells

HAPI cells were not present in the retina or vitreous after LPS activated HAPI cells were injected into the tail vein (figure 7.3). LPS activated HAPI cells were also absent from the optic nerve when injected into the tail vein and there was no ONC (figure 7.4). However, LPS activated HAPI cells were seen along the crush site and the edges of the optic nerve after tail vein injection and ONC injury (figure 7.4). There were no HAPI cells 4 days after injection into the vitreous with (figure 7.6 B) or without (figure 7.6 A) ONC in the retina. At 7 days (figure 7.6 C and D) and 14 days (figure 7.6 E and F), HAPI cells were observed in the vitreous above the ganglion cell layer (GCL) or in the GCL itself. In the

absence of injury, there were no LPS activated HAPI cells seen in the optic nerve (figure 7.7) after intravitreal injection. However, when injected into the vitreous with optic nerve crush, LPS activated HAPI cells and/or other auto-florescent immune cells were seen in the optic nerve (figure 7.7).

7.1.4 RGC Loss after Optic Nerve Crush

Non-injured retinas had 54.97 ± 1.63 RGCs/mm (n=10). Four days after ONC there were 44.90 ± 3.81 RGCs/mm (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$), an expected loss of 18.0 % of RGCs. At 7 days after ONC there was an expected loss of 37.7 % of RGCs with 34.12 ± 1.79 RGCs/mm remaining (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). After 14 days of ONC there was a loss of 65.4 % of RGCs with 18.9 ± 1.05 RGCs/mm remaining (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). These values were used as controls for experiments where activated HAPI cells were introduced into either the vitreous or tail vein.

7.1.5 RGC Loss after Tail Vein HAPI Cell Injection

The RGC density 4 days after the injection of LPS activated HAPI cells in the absence of injury was 56.63 ± 4.00 RGCs/mm (n=6; figure 7.5 A). This was not significantly different from the control (n=10; ANOVA/Tukey's post-hoc test; $p > 0.05$) and from when HAPI cells were injected into the TV without ONC after 4 days (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). After 7 days following injecting LPS activated HAPI cells without ONC there were 54.74 ± 6.05 RGCs/mm (n=6; Figure 7.5 A). This was not significantly different from the

control (n=10; ANOVA/Tukey's post-hoc test; $p > 0.05$) or from when HAPI cells were injected into the TV without activation 7 days later (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). Similarly, there was no difference in the RGC densities when LPS activated HAPI cells were injected14 days previously without injury (55.99 ± 4.43 RGCs/mm; n=6; figure 7.5 A) and from the control (n=10; ANOVA/Tukey's post-hoc test; $p > 0.05$). However, the RGC density was 16.8% greater than the RGC density 14 days after the injection of non-activated HAPI cells into the TV (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). The RGC densities across the 14 day period after injection of LPS activated HAPI cells into the TV were not statistically different from each other and similar to the control (Figure 7.5 A and Table 7.1).

When LPS activated HAPI cells were injected 4 days previously with ONC, there was a RGC density of 56.95 ± 4.03 RGCs/mm (n=6; figure 7.5 B). The RGC density was similar to the uninjured control retina (n=10; ANOVA/Tukey's post-hoc test; $p > 0.05$). The RGC density 7 days after injection of LPS activated HAPI cells accompanied by an ONC was 13.79 ± 7.25 RGCs/mm (n=6; figure 7.5 B). This was 59.5% less than the RGC density 7 days after ONC alone (n=6; ANOVA/Tukey's post-hoc test; $p \leq 0.001$) and 49.6% less than the density 7 days after the injection of HAPI cells and ONC (n=6; ANOVA/Tukey's post-hoc test; $p \leq 0.001$). The RGC density 14 days after LPS activated HAPI cells were injected accompanied by an ONC there were 9.15 ± 4.47 RGCs/mm (n=6; figure 7.5 B). This was 51.7% lower than the RGC density

14 days after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 35.3% lower than the RGC density after HAPI cells were injected with ONC 14 days later (n=6; ANOVA/Tukey's post-hoc test; $p \leq 0.05$).

7.1.6 RGC Loss after Intravitreal HAPI Cell Injection

There were 31.78 ± 5.58 RGCs/mm (n=6; figure 7.8 A) 4 days after LPS activated HAPI cells were injected into the vitreous. This was 42.2% lower than the control (n=10; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 43.5% lower than the RGC density 4 days after injection of HAPI cells into the vitreous (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). Similarly, 7 days after injection of LPS activated HAPI cells there were 34.38 ± 5.7 RGCs/mm (n=6; figure 5.8 A). This was 37.5% lower than the control (n=10; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 40.0% lower than the RGC density 7 days after injection of HAPI cells into the vitreous (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). When LPS activated HAPI cells were injected, the RGC density after 14 days was 33.34 ± 3.97 RGCs/mm (n=6; figure 7.8 A). This RGC density was 39.4% less than the control (n=10; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 27.3% less than the RGC density when HAPI cells were injected into the vitreous without activation (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). When LPS activated HAPI cells were injected into the vitreous without any injury over a 14 day period, the RGC loss was comparable to that seen after 7 days of ONC (n=6; figure 7.8 A and Table 7.4; ANOVA/Tukey's post-hoc test; $p > 0.05$).

The RGC densities after injection of LPS activated HAPI cell into the vitreous with ONC over a 14 day period was lower than the RGC densities after ONC (figure 7.8 B and table 7.5). The RGC density 4 days after LPS activated HAPI cells were injected with an ONC was 13.74 ± 5.48 RGCs/mm (n=6; figure 7.8 B). This was 69.4% lower than the RGC density 4 days after ONC (n=6, ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 63.3% lower than when HAPI cells were injected into the vitreous accompanied by an ONC (n=6, ANOVA/Tukey's post-hoc test, $p \leq 0.001$). When LPS activated HAPI cell were injected 7 days earlier accompanied by an ONC there was a RGC density of 19.71 ± 2.76 RGCs/mm (n=6; figure 7.8 B). This represented a loss of 42.2% of cells when compared to the RGC density 7 days after ONC (n=6, ANOVA/Tukey's post-hoc test, $p \leq 0.001$). However, the RGC loss was statistically not different from when HAPI cells were injected with ONC for 7 days (n=6, ANOVA/Tukey's post-hoc test). The RGC density 14 days after LPS activated HAPI cells were injected accompanied by an ONC was 9.15 ± 4.47 RGCs/mm (n=6; figure 7.8 B). This was 51.7% lower than the RGC density 14 days after ONC (n=6, ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 49.9% lower than when HAPI cells were injected into the vitreous with ONC (n=6, ANOVA/Tukey's post-hoc test, $p \leq 0.001$). The RGC densities were not different from each other over the 14 day period that the LPS activated HAPI cells were injected into the vitreous with ONC (n=6; figure 7.8 B and table 7.5; ANOVA/Tukey's post-hoc test; $p > 0.05$).

7.2 Discussion

This novel method of grafting HAPI cells into the vitreous or injecting them into the tail vein provides an excellent way to study the effect of differentially activated microglia on RGC survival. A problem has been that substances, such as LPS, have been found to activate other cell types in the retina (Fernandes, Silva, Falcão, Brito, & Brites, 2004; Goureau, Hicks, Courtois, & De Kozak, 2002; Jang et al., 2007; Schumann et al., 1998) making it difficult to identify the cellular mechanisms involved in LPS mediated cell death. Previously, microglial activation has been studied *in vitro*, however, this does not help us gain an understanding of how microglial activation effects RGC survival taking into account the complex interplay of different cell types *in vivo*. The method used in the study permitted us to have the strengths of both *in vitro* and *in vivo* approaches. We were able to activate the microglia cells *in vitro* and introduce them *in vivo*, without having the side effects of the drug *in vivo*.

The LAL assay was used to determine the level of LPS in the injection solution to rule out the possibility that residual LPS was accompanying the injected cells. The same absorbance at 545 nm was determined for both solutions and it equated to endotoxin levels of 0.104 EU/mL (approximately 0.0104 ng/mL) in the HAPI cell solution and 0.092 EU/mL (approximately 0.0095 ng/mL) in the LPS activated HAPI cell solution. This demonstrated that there was no residual LPS in the LPS activated HAPI cell solution than the HAPI cell solution itself.

It is commonly thought that the blood-brain barrier (BBB) controls the entry of immune cells into the CNS and that resident microglia perform the action that is normally the responsibility of the immune system (Aguzzi, Barres, & Bennett, 2013). However, this concept is now being challenged by studies showing that certain immune cells are able to enter the CNS and that there is communication between the CNS and immune system across the BBB (Crane & Liversidge, 2008). Previously, we have shown that HAPI cells injected into the systemic system via the tail vein were able to migrate to the optic nerve if there was an injury signal present. Some studies showed that injured neurons released purine nucleotides such as ATP, ADP, and UTP, to activate microglial cells and help them migrate to the site of injury (Davalos et al., 2005; Nimmerjahn, Kirchhoff, & Helmchen, 2005). This may be one of the many possible mechanisms used by injured neurons to communicate with the immune system. However, neurons are not the only cells that can attract cells from the periphery. Microglia have been shown to attract monocytes to the CNS by up-regulating expression of MCP-1 (D'Mello, Le, & Swain, 2009). Previously, we showed that HAPI cells migrate to the injury site in the optic nerve when HAPI cells were injected into the tail vein but not the vitreous (Chapter 3). Similarly, when LPS activated HAPI cells were injected into the tail vein they migrated to the crush site in the optic nerve. However, there were HAPI cells as well as auto-fluorescent immune cells present in the optic nerve when LPS activated HAPI cells were injected into the vitreous. This suggested that signals are required for microglial

cells and immune cells to migrate to the area of injury and that hyper-activated microglial cells attract systemic immune cells to the injury site. Dendritic cells are seen in ALS spinal cord tissue and T-cells appear at the end stages in mSOD1 mice (Alexianu, Kozovska, & Appel, 2001; Henkel et al., 2004). In addition, microglial activation may attract monocyte derived macrophages from the systemic system to terminate microglial activity (London et al., 2011; Shechter et al., 2009). However, much remains unknown about the differential function of monocyte derived macrophages and microglia since they are hard to distinguish from each other (Jung & Schwartz, 2012).

The migration of microglia and immune cells to the injury site was correlated with the loss of RGCs in the retina. At 4 days, after LPS activated HAPI cells were injected into the tail vein accompanied by an ONC, there was greater cell survival than when there was ONC alone or HAPI cells were injected with ONC but without LPS activation. However 7 days after injury, the RGC density returned to expected values. This suggests that when hyper-activated HAPI cells were injected into the tail vein, they were beneficial to neuronal survival early in the injury. This finding agrees with some of the results from studies done with spinal cord injury models. It has been suggested that the microglial response after injury is required for repair early in the injury, however, the beneficial effect of microglia was lost later in the injury (Shechter et al., 2009). Also, the recruitment of monocyte derived cells was necessary to terminate the microglial response (Shechter et al., 2009). Perhaps the hyper-activated state

is less prone to modulation by other cell types, resulting in a more deleterious effect later in injury.

The amount of RGC that survived after hyper-activated HAPI cells were grafted into the vitreous contrasted the survival of RGCs after tail vein injection. LPS activated HAPI cells were seen in the vitreous and the ganglion cell layer with or without injury after 7 days. In both cases, there was increased RGC loss. When LPS activated HAPI cells were injected into the vitreous without injury, there was a 37 – 42% loss in RGC compared to controls. This was different from our previous study that demonstrated no loss of RGCs after HAPI cells were injected into the vitreous within the first 14 days. This suggests that LPS activation resulted in a pro-inflammatory response, even in the absence of neuronal injury, resulting in the loss of RGCs when hyper-activated microglia are present in the vitreous. The anatomical location (cell body/optic nerve or local/systemic) where the microglia act may affect neuronal survival. A similar finding was observed over several weeks after middle cerebral artery occlusion where there was a different pattern of microglial activation in the subventricular zone (SVZ) compared to the striatum (Thored et al., 2008). The microglia in the SVZ were more ramified and in a resting state, while in the striatum they were more amoeboid and in an activated state (Thored et al., 2008). Therefore, the properties of the different anatomical areas may modulate the response of the microglial cells.

We also observed increased RGC loss after injection of LPS activated HAPI cells into the vitreous accompanied by an ONC. The RGC density was 42 – 69% lower than that observed after ONC alone over the 14 day period. When compared to the RGC density after HAPI cells were injected into the vitreous, there were 63% less RGCs at day 4 and 50% less at day 14. Therefore, LPS induced hyper-activity in HAPI cells resulted in a pro-inflammatory response that is detrimental to RGC survival when injected into the vitreous. This was similar to other findings that showed that LPS activation of microglial cells led to a strong pro-inflammatory response due to the release of cytokines and superoxides (Chen et al., 2012; Lu, Yeh, & Ohashi, 2008; Qin et al., 2005). LPS may put macrophages into M1 activated phenotype, which is commonly associated with cytotoxicity (Martinez, Sica, Mantovani, & Locati, 2008). However, our results from the tail vein injections show that LPS activated HAPI cells are not always cytotoxic to RGCs and may help promote survival early in the injury. It has been shown that repeated low-dose exposures to LPS can lead to neuroprotection after CNS injury and resulted in less inflammation (Chen et al., 2012). In addition, zymosan activated macrophages have been shown to stimulate axonal regeneration, although it can also be cytotoxic, emphasizing how easily the balance between pro-inflammatory and neuroprotective states can be changed (Gensel et al., 2009). It has been very difficult to determine what the best microglial response is after injury (Aguzzi et al., 2013). The M1 activation state is thought to be neurotoxic but may help promote axonal regeneration, while the M2

state is hard to maintain in an injured environment (Aguzzi et al., 2013). However, it seems clear that attempting to promote the M2 phenotype and controlling the time and location of action by microglia would help tip the balance to a neuroprotective outcome.

It was noted with retinas that were treated with LPS activated HAPI cells injected into the vitreous that there was inflammation in some eyes (figure 6). Eyes in the same conditions, such as 4 days after injection of LPS activated HAPI cells, had varying degrees of inflammation from high (figure 6A) to none (figure 6B). This varying level of inflammation may lead to highly variable in cell counts, such that the highly inflamed eyes has significantly fewer RGCs/mm (table 7). There may be individual differences in the susceptibility to inflammation and reducing this type of inflammation may result in increased cell survival. After LPS activation of HAPI cells there may be increased immune infiltrate due to a weakening of the BBB. It has been suggested that inflammatory cells increased the level of MMP-9 that led to BBB disruption. Immune cells may increase levels of MMP-9 by directly releasing MMP-9, activating transcription of MMP-9 through the release of cytokines (such as IL-6 and TNFα), or by increasing levels of reactive oxygen species (ROS) leading to activation of proMMP-9 by oxidation or N-nitrosylation (Lehner et al., 2011). Previous studies have shown that hyper-activating HAPI cells with LPS results in an increase in cytokine production and ROS (Cheepsunthorn, Radov, Menzies, Reid, & Connor, 2001; Horvath, Nutile-McMenemy, Alkaitis, & DeLeo, 2008; Qin et al., 2005;

Zheng et al., 2012). These may contribute to the breakdown of the BBB, increasing the amount of immune cells infiltrate resulting in overt inflammation. However, it is important to note that not all eyes were inflamed suggesting that there may be unknown individual protective factors that contribute to lower inflammation by the same treatment.

Our results show that LPS activated HAPI cells were neuroprotective early in injury when injected into the tail vein accompanied by an ONC, however, are pro-inflammatory later in the injury. When LPS activated HAPI cells were injected into the vitreous, it resulted in a pro-inflammatory response and increased RGC death. This suggests that the location where the microglia act and the time course of the injury determines if microglia have a neuroprotective or cytotoxic phenotype. The hyper-activation of microglial cells may result in the disruption of the BBB that increases immune infiltration into the CNS. Classically, the CNS has been considered to be an immune privileged area, however, this study demonstrates that the CNS has interaction with the systemic system and that immune cells can affect neuronal survival. Identifying the conditions that contribute to microglia having a neuroprotective or cytotoxic phenotype will help provide insight into the role of neuroinflammation in neurodegenerative diseases and lead to new potential therapeutic targets.

7.3 Figures and Tables

Figure 7.1: Co-localization of Fluorogold (gold) with Brn3a (green) in 12 µm thick frozen transverse sections of rat retinas. (A) Retina with fluorogold 7 days after optic nerve crush. Some cells are labelled with fluorogold but not Brn3a (arrowhead), these may be microglia. Other retinal ganglion cells labelled with fluorogold are co-localized with Brn3a (arrows). **(B)** Retina immunostained with Brn3a 7 days after ONC. **(C)** Fluorogold containing RGCs immunoreactive for Brn3a 7 day after ONC. **(D)** Retina with fluorogold 14 days after optic nerve crush. **(E)** Retina immunostained with Brn3a 14days after ONC. Some cells are immunoreactive Brn3a but not Fluorogold (dotted circle). These cells also have a spotted staining pattern similar to some background staining areas. **(F)** Fluorogold containing RGCs immunoreactive for Brn3a 14 day after ONC. **(G)** Retina with fluorogold. Some cells are labelled with fluorogold but not Brn3a (arrowhead), these may be microglia. Other retinal ganglion cells labelled with fluorogold are co-localized with Brn3a (arrows). **(H)** Retina immunostained with Brn3a. **(I)** Fluorogold containing RGCs immunoreactive for Brn3a. About 90% of RGCs are immunoreactive for Brn3a.

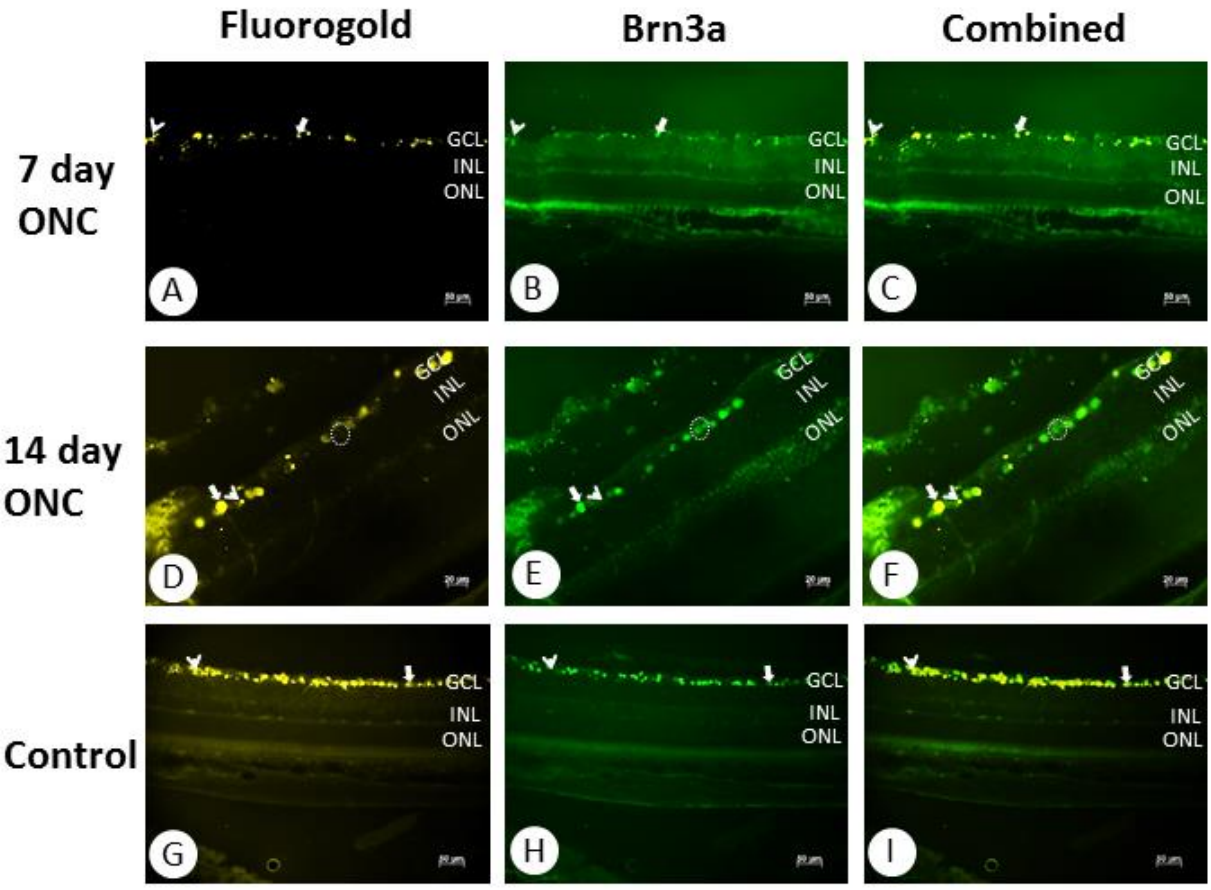

Fluorogold
Brn3a
Combined
7 day ONC
14 day ONC
Control
GCL
INL
ONL
A
B
C
D
E
F
G
H
I

Figure 7.2: Endotoxin levels in the HAPI cell injection solution. The LAL endotoxin assay kit was used to determine the levels of endotoxin/LPS in the HAPI cell solution used for injection. The line of best fit was created from a blank and endotoxin standard from concentrations of 0.01-0.1 EU/mL. After the absorbance at 545 nm was determined for the HAPI cell solution (green square) and LPS activated HAPI cells (orange diamond). This numbers were used to determine the endotoxin concentration of the solution, which was calculated to be 0.104 EU/mL for HAPI cells and 0.095 EU/mL for LPS activated HAPI cells.

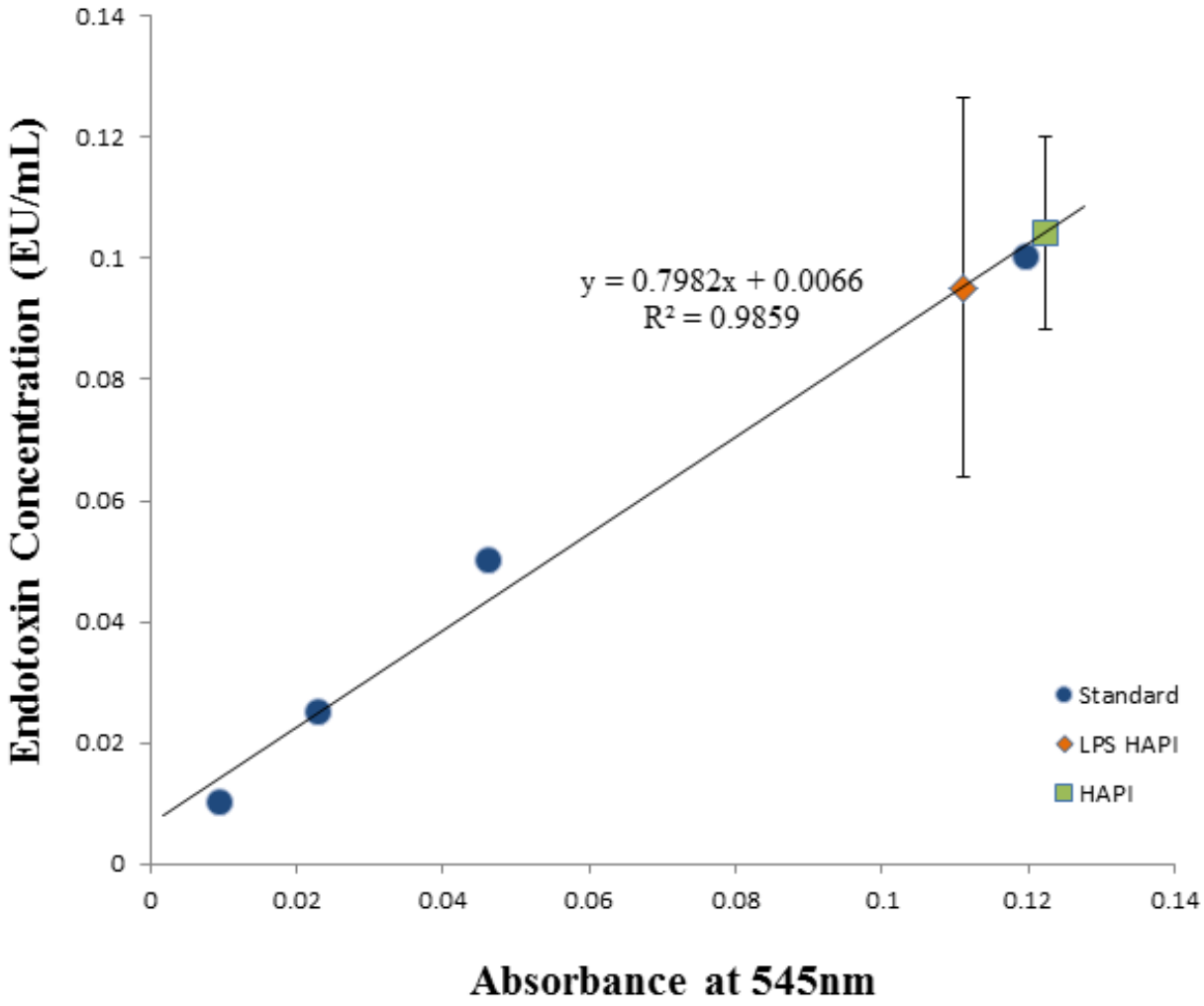

Endotoxin Concentration (EU/mL)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
y = 0.7982x + 0.0066
R² = 0.9859
Standard
LPS HAPI
HAPI
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
Absorbance at 545nm

Figure 7.3: Brn3a immunoreactive RGCs (green) survival after tail vein injection of LPS activated HAPI cells labelled with WGA-TR (red) in 12 µm thick frozen transverse sections of rat retinas. (A) Retinas collected 4 days after LPS activated HAPI cells were injected. **(B)** Retinas collected 4 days after LPS activated HAPI cells were injected and ONC. **(C)** Retinas collected 7 days after LPS activated HAPI cells were injected. **(D)** Retinas collected 7 days after LPS activated HAPI cells were injected and ONC. **(E)** Retinas collected 14 days after LPS activated HAPI cells were injected. **(F)** Retinas collected 14 days after LPS activated HAPI cells were injected. No HAPI cells were seen in retinal sections after tail vein injection.

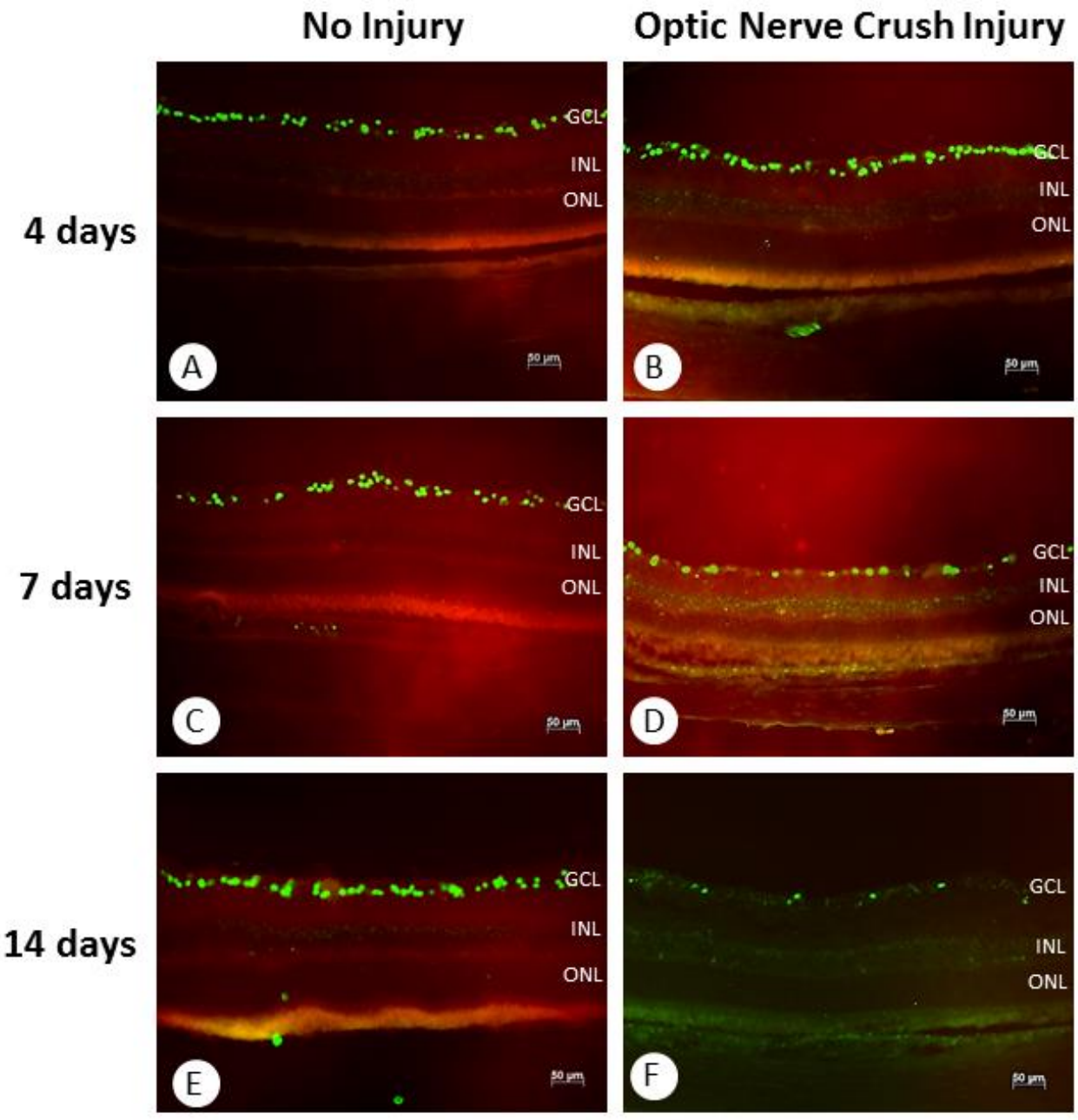

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
GCL
INL
ONL
A
B
C
D
E
F
50 µm

Figure 7.4: 12 µm thick frozen sections of optic nerve that had LPS activated HAPI cells (red) injected into the tail vein with and without optic nerve crush. (A) 4 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(B)** 4 days after injection and with ONC there were HAPI cells present in the optic nerve at the crush site. **(C)** 7 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(D)** 7 days after injection and with ONC there were HAPI cells present in the optic nerve at the crush site radiating outward. (**E)** 14 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(F)** 14 days after injection and with ONC there were HAPI cells present in the optic nerve at the crush site radiating outward.

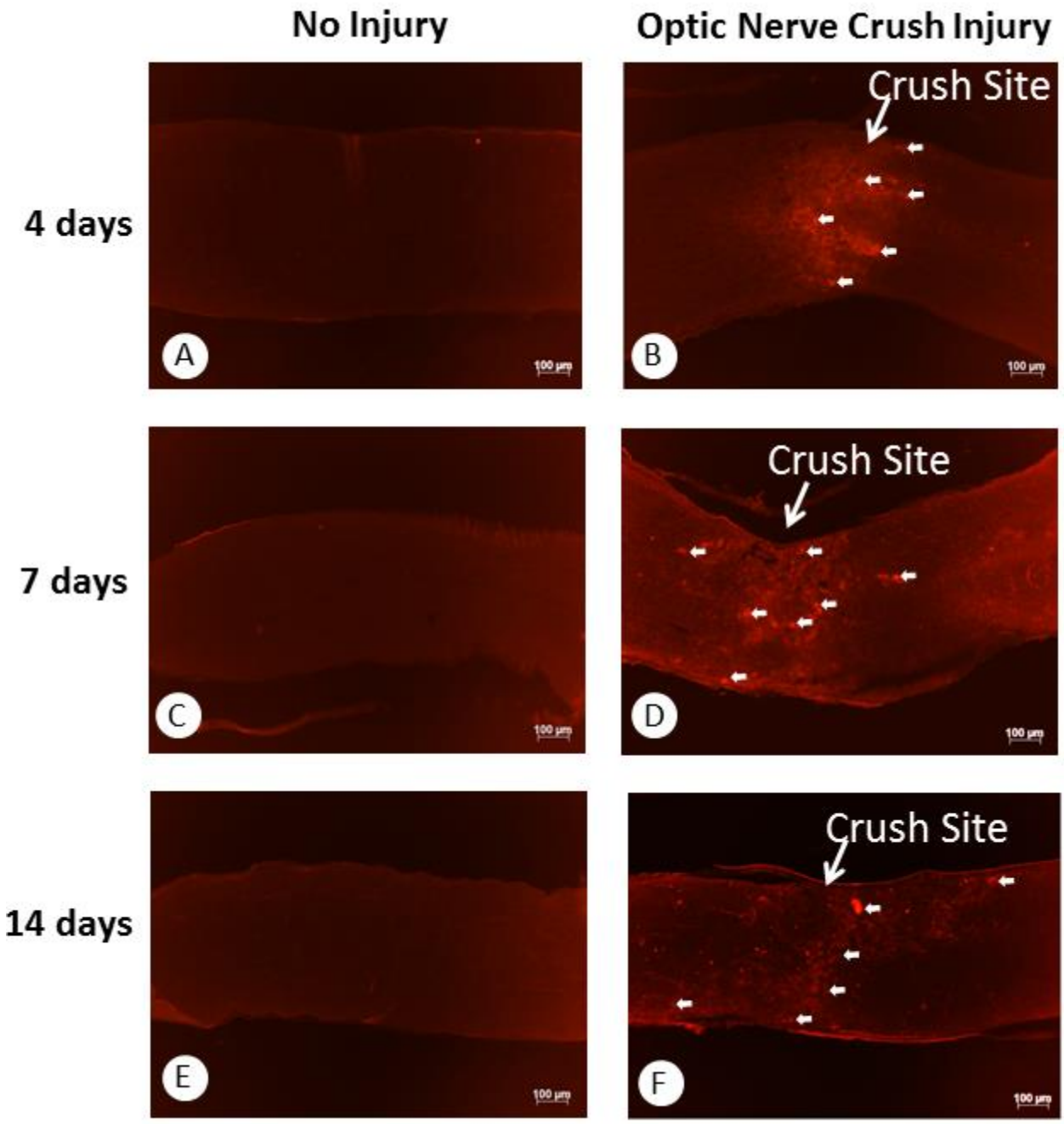
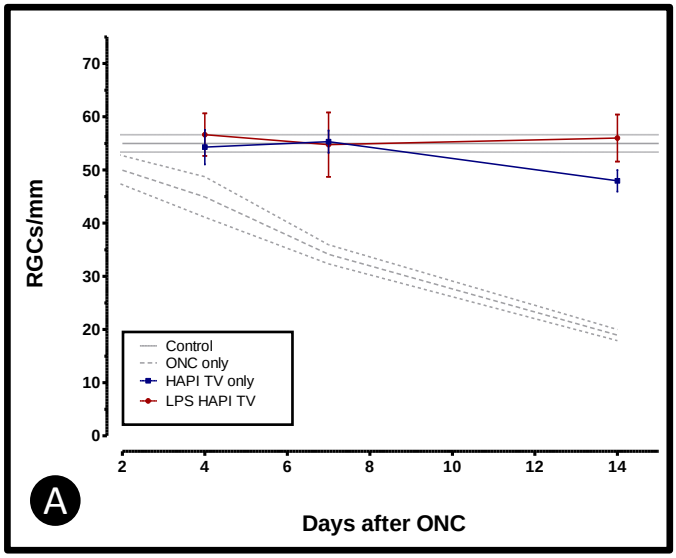
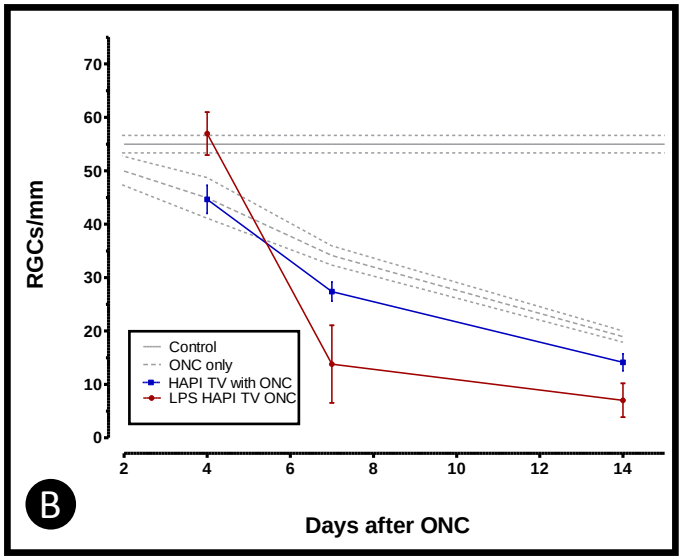

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
Crush Site
Crush Site
Crush Site
A
B
C
D
E
F
100 µm

Figure 7.5: RGC survival 14 days after tail vein injection of LPS activated HAPI cells. (A) RGC survival after injection of untreated HAPI cells into the tail vein (blue square) was not statistically different from the control except at 14 days. RGC survival after injection of LPS activated HAPI cells into the vitreous (red circles) was not statistically different from the control. The RGC density was not different across the 14 day period. When HAPI cells are activated with LPS without ONC there was a statistically greater survival of RGCs 14 days after injection as compared to RGC survival after untreated HAPI cell injection. **(B)** RGC density 4 days after injection of LPS activated HAPI cells with ONC (red circle) was not different from control (solid grey line). However, 7 days after ONC and injection of LPS activated HAPI cells, the RGC density was lower than the RGC density 7 days after just ONC. Therefore, there was greater survival of RGCs early in the injury and less late in the injury. **(C)** 7 days after injection of LPS activated HAPI cells into the tail vein with ONC (blue square) there was greater cell death than when LPS activated HAPI cells were injected without ONC (red circle).

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
Control
ONC only
HAPI TV only
LPS HAPI TV
A


RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
Control
ONC only
HAPI TV with ONC
LPS HAPI TV ONC
B

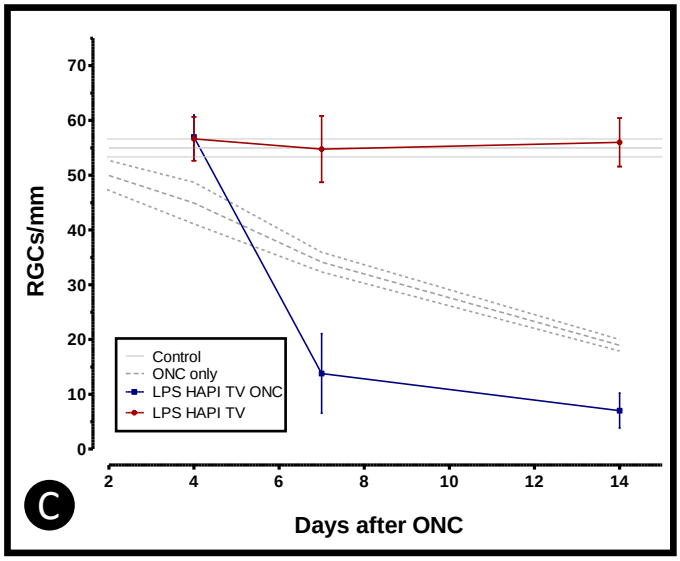

RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after ONC
Control
ONC only
LPS HAPI TV ONC
LPS HAPI TV
C

Table 7.1: Tukey's Multiple Comparison Test after ANOVA statistical analysis of HAPI cells injected into the tail vein with and without LPS activation. The analysis is for data represented in figure 7.5.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI TV (4 days) vs LPS HAPI TV (7 days)	No	ns
LPS HAPI TV (4 days) vs LPS HAPI TV (14days)	No	ns
LPS HAPI TV (4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI TV (4 days) vs HAPI TV (4 days)	No	ns
LPS HAPI TV (4 days) vs HAPI TV (7 days)	No	ns
LPS HAPI TV (4 days) vs HAPI TV (14 days)	No	P ≤ 0.01
LPS HAPI TV (4 days) vs control	No	ns
LPS HAPI TV (7 days) vs LPS HAPI TV (14days)	No	ns
LPS HAPI TV (7 days) vs ONC 7 days	Yes	P ≤ 0.001
LPS HAPI TV (7 days) vs HAPI TV (7 days)	No	ns
LPS HAPI TV (7 days) vs control	No	ns
LPS HAPI TV (14days) vs ONC 14 days	Yes	P ≤ 0.001
LPS HAPI TV (14days) vs HAPI TV (14 days)	Yes	P ≤ 0.001
LPS HAPI TV (14days) vs control	No	ns
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.001
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs HAPI TV (4 days)	Yes	P ≤ 0.01
ONC 4 days vs HAPI TV (14 days)	No	ns
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs HAPI TV (7 days)	Yes	P ≤ 0.001
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 14 days vs HAPI TV (14 days)	Yes	P ≤ 0.001
ONC 14 days vs control	Yes	P ≤ 0.001
HAPI TV (4 days) vs HAPI TV (7 days)	No	ns
HAPI TV (4 days) vs HAPI TV (14 days)	No	ns
HAPI TV (4 days) vs control	No	ns
HAPI TV (7 days) vs HAPI TV (14 days)	Yes	P ≤ 0.05
HAPI TV (7 days) vs control	No	ns
HAPI TV (14 days) vs control	Yes	P ≤ 0.05

Table 7.2: Tukey's Multiple Comparison Test after ANOVA statistical analysis of HAPI cells injected into the tail vein with and without LPS activation after optic nerve crush. The analysis is for data represented in figure 7.5b.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI TV ONC(4 days) vs LPS HAPI TV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs LPS HAPI TV ONC(14days)	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs HAPI TV ONC (4 days)	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs control	No	ns
LPS HAPI TV ONC (7 days) vs LPS HAPI TV ONC(14days)	No	ns
LPS HAPI TV ONC (7 days) vs ONC 7 days	Yes	P ≤ 0.001
LPS HAPI TV ONC (7 days) vs HAPI TV ONC (4 days)	Yes	P ≤ 0.001
LPS HAPI TV ONC (7 days) vs HAPI TV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI TV ONC (7 days) vs control	Yes	P ≤ 0.001
LPS HAPI TV ONC(14days) vs ONC 14 days	Yes	P ≤ 0.001
LPS HAPI TV ONC(14days) vs HAPI TV ONC (14 days)	Yes	P ≤ 0.05
LPS HAPI TV ONC(14days) vs control	Yes	P ≤ 0.001
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.001
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs HAPI TV ONC (4 days)	No	ns
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs HAPI TV ONC (7 days)	Yes	P ≤ 0.01
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 14 days vs HAPI TV ONC (14 days)	Yes	P ≤ 0.01
ONC 14 days vs control	Yes	P ≤ 0.001
HAPI TV ONC (4 days) vs HAPI TV ONC (7 days)	Yes	P ≤ 0.001
HAPI TV ONC (4 days) vs HAPI TV ONC (14 days)	Yes	P ≤ 0.001
HAPI TV ONC (4 days) vs control	Yes	P ≤ 0.001
HAPI TV ONC (7 days) vs HAPI TV ONC (14 days)	Yes	P ≤ 0.001
HAPI TV ONC (7 days) vs control	Yes	P ≤ 0.001
HAPI TV ONC (14 days) vs control	Yes	P ≤ 0.001

Table 7.3: Tukey's Multiple Comparison Test after ANOVA statistical analysis of LPS activated HAPI cells injected into the tail vein with or without optic nerve crush. The analysis is for data represented in figure 7.5c.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI TV (4 days) vs LPS HAPI TV (7 days)	No	ns
LPS HAPI TV (4 days) vs LPS HAPI TV (14days)	No	ns
LPS HAPI TV (4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI TV (4 days) vs LPS HAPI TV ONC(4 days)	No	ns
LPS HAPI TV (4 days) vs control	No	ns
LPS HAPI TV (7 days) vs LPS HAPI TV (14days)	No	ns
LPS HAPI TV (7 days) vs ONC 7 days	Yes	P ≤ 0.001
LPS HAPI TV (7 days) vs LPS HAPI TV ONC(4 days)	No	ns
LPS HAPI TV (7 days) vs LPS HAPI TV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI TV (7 days) vs control	No	ns
LPS HAPI TV (14days) vs ONC 14 days	Yes	P ≤ 0.001
LPS HAPI TV (14days) vs LPS HAPI TV ONC(4 days)	No	ns
LPS HAPI TV (14days) vs LPS HAPI TV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI TV (14days) vs LPS HAPI TV ONC(14days)	Yes	P ≤ 0.001
LPS HAPI TV (14days) vs control	No	ns
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.001
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs LPS HAPI TV ONC(4 days)	Yes	P ≤ 0.001
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs LPS HAPI TV ONC (7 days)	No	ns
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 14 days vs LPS HAPI TV ONC(14days)	Yes	P ≤ 0.01
ONC 14 days vs control	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs LPS HAPI TV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs LPS HAPI TV ONC(14days)	Yes	P ≤ 0.001
LPS HAPI TV ONC(4 days) vs control	No	ns
LPS HAPI TV ONC (7 days) vs LPS HAPI TV ONC(14days)	Yes	P ≤ 0.001
LPS HAPI TV ONC (7 days) vs control	Yes	P ≤ 0.001
LPS HAPI TV ONC(14days) vs control	Yes	P ≤ 0.001

Figure 7.6: Brn3a immunoreactive RGCs survival after intravitreal injection of LPS activated HAPI cells in 12 µm thick frozen transverse sections of rat retinas. (A) Retinas collected 4 days after LPS activated HAPI cells were injected. **(B)** Retinas collected 4 days after LPS activated HAPI cells were injected and ONC. **(C)** Retinas collected 7 days after LPS activated HAPI cells were injected. **(D)** Retinas collected 7 days after LPS activated HAPI cells were injected and ONC. **(E)** Retinas collected 14 days after LPS activated HAPI cells were injected. **(F)** Retinas collected 14 days after LPS activated HAPI cells were injected. HAPI cells (arrows) can be seen in both crushed and not crushed conditions 7 days after injection and onward.

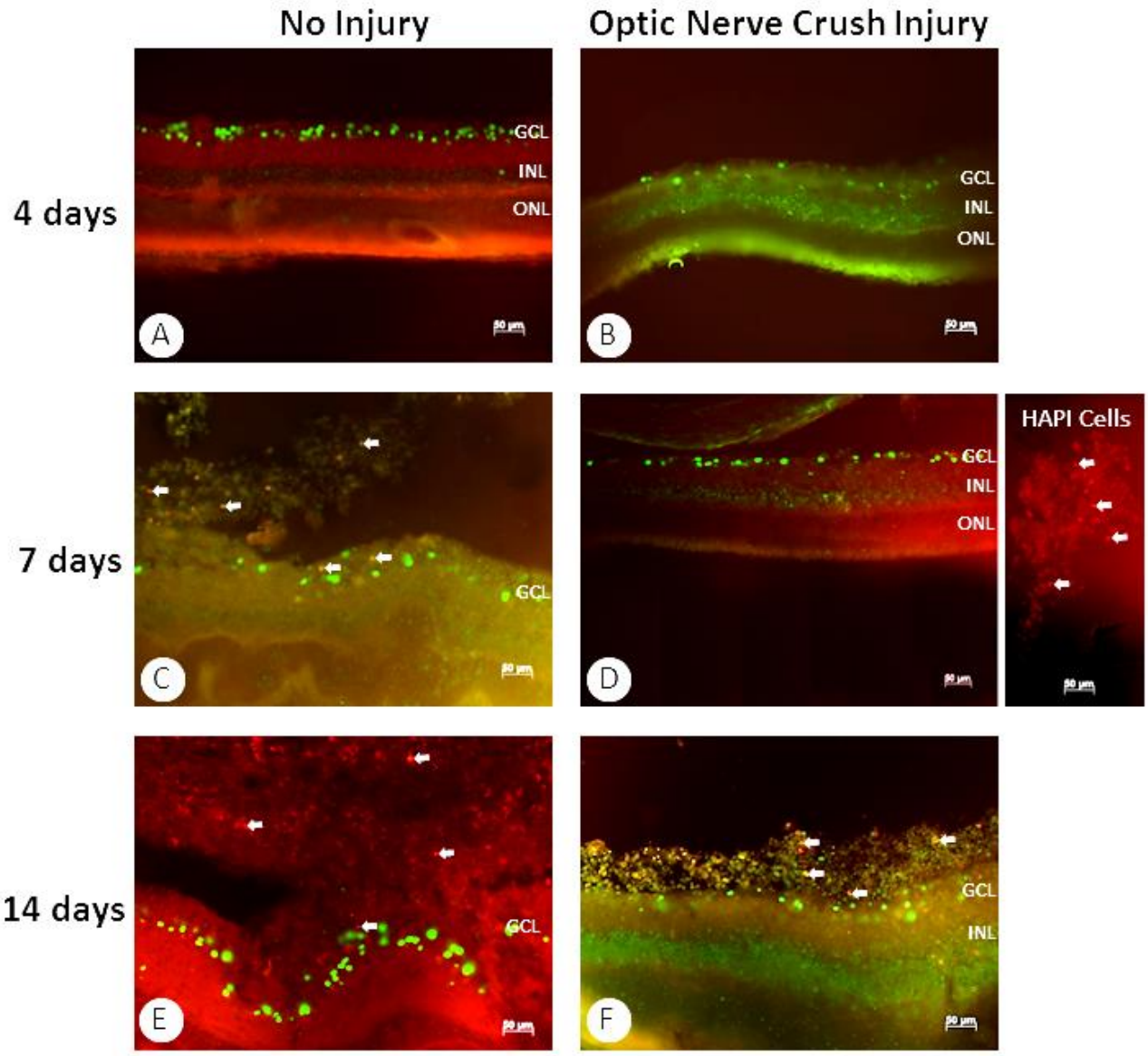

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
GCL
INL
ONL
A
B
C
D
E
F
HAPI Cells

Figure 7.7: 12 µm thick frozen sections of optic nerve that had LPS activated HAPI cells (red) injected into the vitreous with and without optic nerve crush. (A) 4 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(B)** 4 days after injection and with ONC there were HAPI cells and auto-fluorescent immune cells present in the optic nerve at the crush site. **(C)** 7 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(D)** 7 days after injection and with ONC there were HAPI cells and auto-fluorescent immune cells present in the optic nerve at the crush site radiating outward. (**E)** 14 days after injection but in the absence of ONC there were no HAPI cells present in the optic nerve. **(F)** 14 days after injection and with ONC there were HAPI cells and auto-fluorescent immune cells present in the optic nerve at the crush site radiating outward.

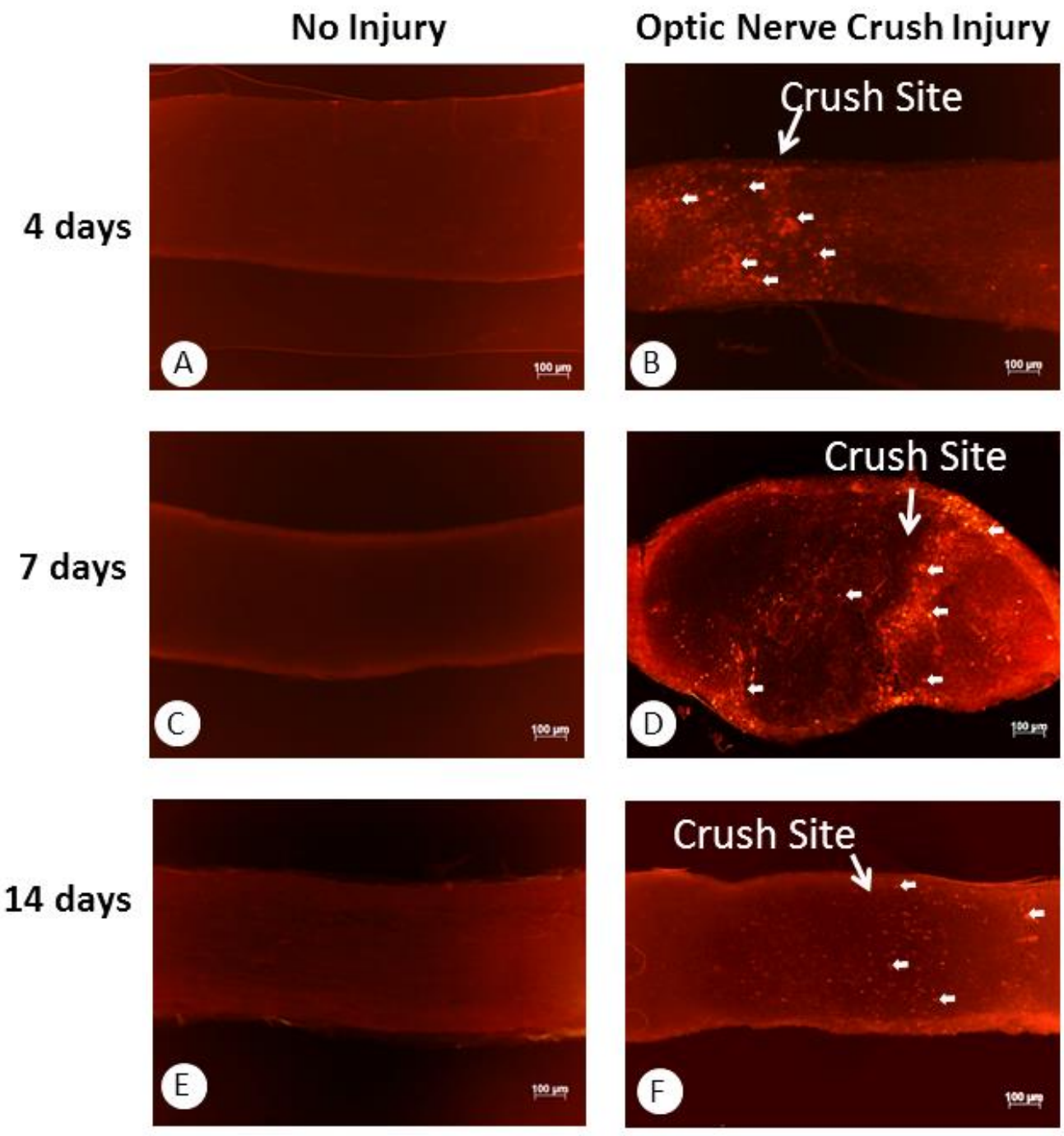
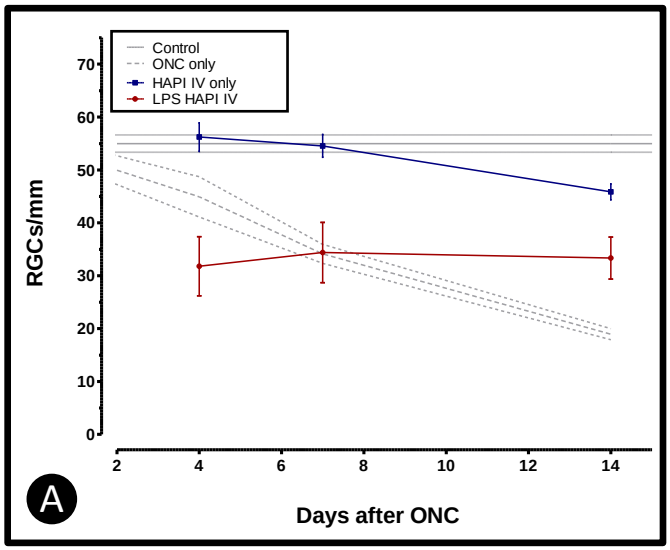
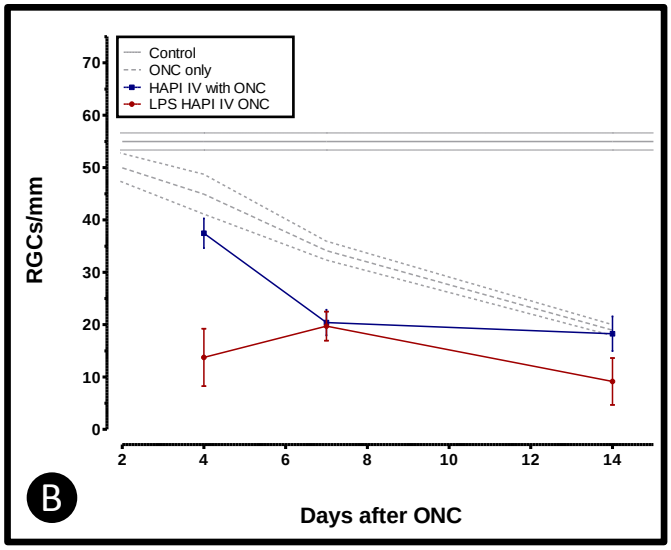

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
Crush Site
Crush Site
Crush Site
A
B
C
D
E
F
100 µm

Figure 7.8: RGCs survival 14 days after intravitreal injection of LPS activated HAPI cells. (A) HAPI cells injected into the vitreous with or without LPS activation. RGC density after injection of LPS activated HAPI cells (red circles) were not statistically different from the RGC density 7 days after ONC (grey dotted line). When HAPI cells were injected without LPS activation (blue squares), the RGC density was not statistically different than control except at 14 days. Injection of hyper-activated HAPI cells leads to greater cell death. **(B)** HAPI cells injected into the vitreous with or without LPS activation after ONC. RGC density after injection of LPS activated HAPI cells and ONC red circles) were lower than the RGC density after ONC alone (grey dotted line). When HAPI cells were injected into the vitreous without LPS activation and with ONC (blue squares), there was greater RGC survival than when LPS activated HAPI cells are injected with ONC (except for at 7 days after injection). **(C)** RGC density after injection of LPS activated HAPI cells and ONC was lower than when LPS HAPI cells were injected without ONC. Injection of LPS activated HAPI cells into the vitreous led to increase RGC death.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


Control
ONC only
HAPI IV only
LPS HAPI IV
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after ONC
A


Control
ONC only
HAPI IV with ONC
LPS HAPI IV ONC
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after ONC
B

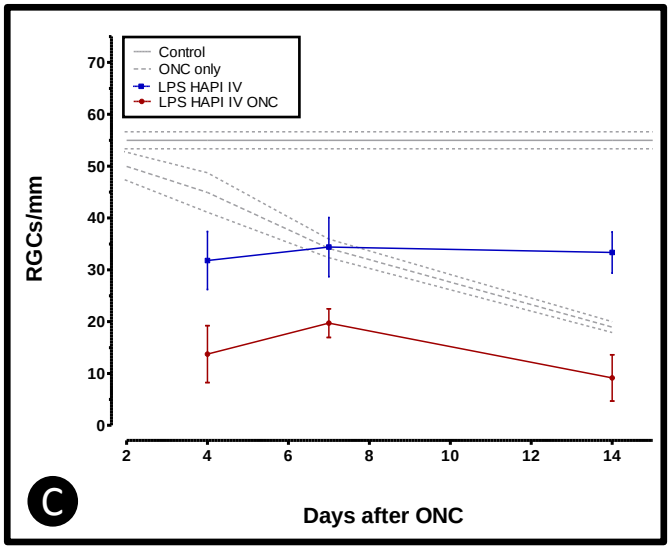

Control
ONC only
LPS HAPI IV
LPS HAPI IV ONC
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after ONC
C

Table 7.4: Tukey's Multiple Comparison Test after ANOVA statistical analysis of HAPI cells injected into the vitreous with and without LPS activation. The analysis is for data represented in figure 7.8A.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI IV (4 days) vs LPS HAPI IV (7 days)	No	ns
LPS HAPI IV (4 days) vs LPS HAPI IV (14 days)	No	ns
LPS HAPI IV (4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI IV (4 days) vs ONC 7 days	No	ns
LPS HAPI IV (4 days) vs HAPI IV (4 days)	Yes	P ≤ 0.001
LPS HAPI IV (4 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV (7 days) vs LPS HAPI IV (14 days)	No	ns
LPS HAPI IV (7 days) vs ONC 7 days	No	ns
LPS HAPI IV (7 days) vs HAPI IV (7 days)	Yes	P ≤ 0.001
LPS HAPI IV (7 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV (14 days) vs ONC 7 days	No	ns
LPS HAPI IV (14 days) vs ONC 14 days	Yes	P ≤ 0.001
LPS HAPI IV (14 days) vs HAPI IV (14 days)	Yes	P ≤ 0.05
LPS HAPI IV (14 days) vs control	Yes	P ≤ 0.001
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.001
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs HAPI IV (14 days)	No	ns
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 14 days vs control	Yes	P ≤ 0.001
HAPI IV (4 days) vs HAPI IV (7 days)	No	ns
HAPI IV (4 days) vs HAPI IV (14 days)	Yes	P ≤ 0.001
HAPI IV (4 days) vs control	No	ns
HAPI IV (7 days) vs HAPI IV (14 days)	Yes	P ≤ 0.01
HAPI IV (7 days) vs control	No	ns
HAPI IV (14 days) vs control	Yes	P ≤ 0.001

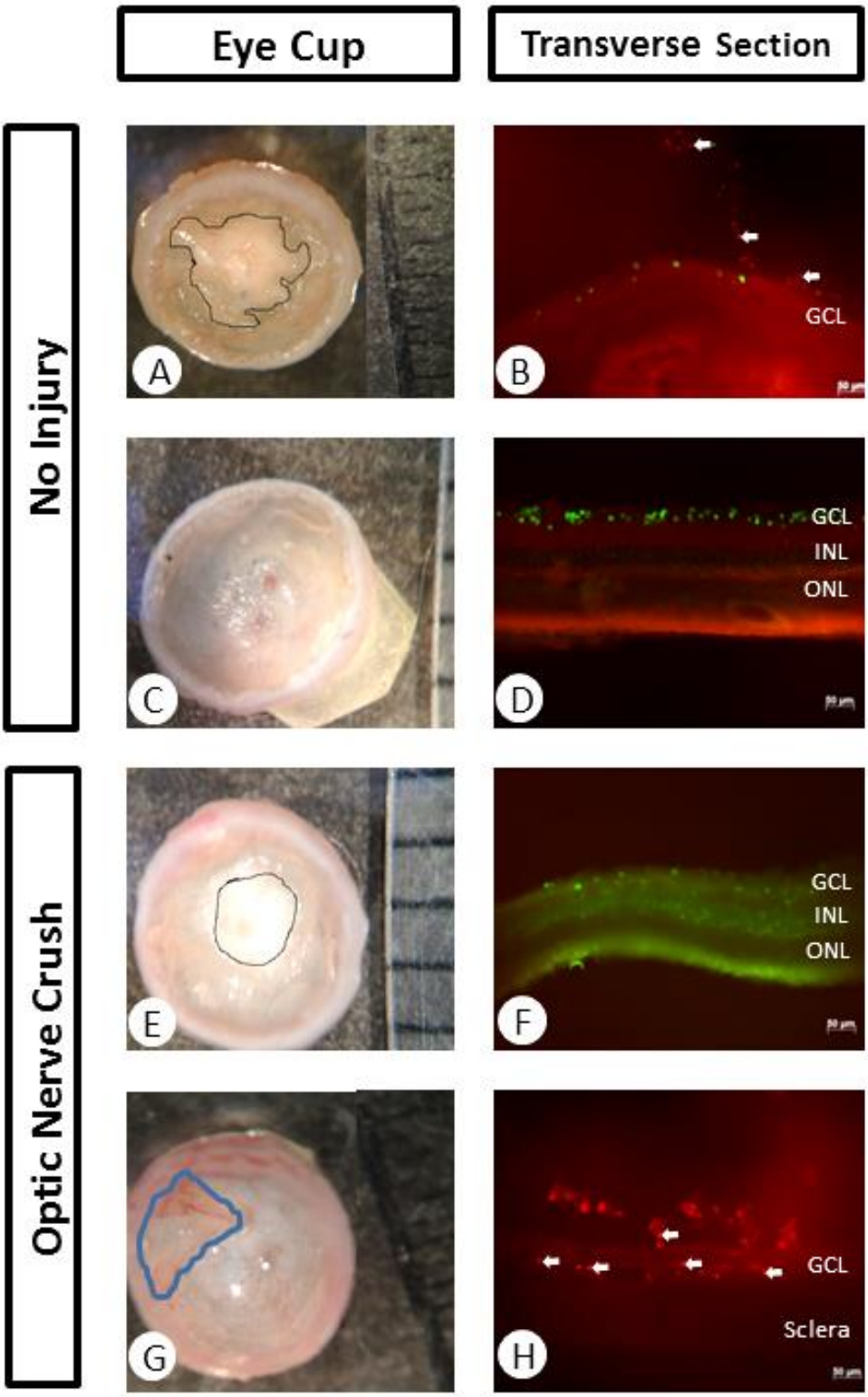
Table 7.5: Tukey's Multiple Comparison Test after ANOVA statistical analysis of HAPI cells injected into the vitreous with and without LPS activation after optic nerve crush. The analysis is for data represented in figure 7.8B.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI IV ONC (4 days) vs LPS HAPI IV ONC (7 days)	No	ns
LPS HAPI IV ONC (4 days) vs LPS HAPI IV ONC (14 days)	No	ns
LPS HAPI IV ONC (4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI IV ONC (4 days) vs ONC 14 days	No	ns
LPS HAPI IV ONC (4 days) vs HAPI IV ONC (4 days)	Yes	P ≤ 0.001
LPS HAPI IV ONC (4 days) vs HAPI IV ONC (7 days)	No	ns
LPS HAPI IV ONC (4 days) vs HAPI IV ONC (14 days)	No	ns
LPS HAPI IV ONC (4 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV ONC (7 days) vs LPS HAPI IV ONC (14 days)	No	ns
LPS HAPI IV ONC (7 days) vs ONC 7 days	Yes	P ≤ 0.001
LPS HAPI IV ONC (7 days) vs ONC 14 days	No	ns
LPS HAPI IV ONC (7 days) vs HAPI IV ONC (7 days)	No	ns
LPS HAPI IV ONC (7 days) vs HAPI IV ONC (14 days)	No	ns
LPS HAPI IV ONC (7 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV ONC (14 days) vs ONC 14 days	Yes	P ≤ 0.05
LPS HAPI IV ONC (14 days) vs HAPI IV ONC (14 days)	Yes	P ≤ 0.05
LPS HAPI IV ONC (14 days) vs control	Yes	P ≤ 0.001
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.001
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs HAPI IV ONC (4 days)	Yes	P ≤ 0.01
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs HAPI IV ONC (4 days)	No	ns
ONC 7 days vs HAPI IV ONC (7 days)	Yes	P ≤ 0.001
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 14 days vs HAPI IV ONC (7 days)	No	ns
ONC 14 days vs HAPI IV ONC (14 days)	No	ns
ONC 14 days vs control	Yes	P ≤ 0.001
HAPI IV ONC (4 days) vs HAPI IV ONC (7 days)	Yes	P ≤ 0.001
HAPI IV ONC (4 days) vs HAPI IV ONC (14 days)	Yes	P ≤ 0.001
HAPI IV ONC (4 days) vs control	Yes	P ≤ 0.001
HAPI IV ONC (7 days) vs HAPI IV ONC (14 days)	No	ns
HAPI IV ONC (7 days) vs control	Yes	P ≤ 0.001
HAPI IV ONC (14 days) vs control	Yes	P ≤ 0.001

Table 7.6: Tukey's Multiple Comparison Test after ANOVA statistical analysis of LPS activated HAPI cells injected into the vitreous with or without optic nerve crush. The analysis is for data represented in figure 7.8C.

Tukey's Multiple Comparison Test	Significant? P < 0.05?	Summary
LPS HAPI IV (4 days) vs LPS HAPI IV (7 days)	No	ns
LPS HAPI IV (4 days) vs LPS HAPI IV (14 days)	No	ns
LPS HAPI IV (4 days) vs ONC 4 days	Yes	P ≤ 0.001
LPS HAPI IV (4 days) vs ONC 7 days	No	ns
LPS HAPI IV (4 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV (4 days) vs LPS HAPI IV ONC (4 days)	Yes	P ≤ 0.01
LPS HAPI IV (4 days) vs LPS HAPI IV ONC (7 days)	Yes	P ≤ 0.05
LPS HAPI IV (4 days) vs LPS HAPI IV ONC (14 days)	Yes	P ≤ 0.001
LPS HAPI IV (7 days) vs LPS HAPI IV (14 days)	No	ns
LPS HAPI IV (7 days) vs ONC 7 days	No	ns
LPS HAPI IV (7 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV (7 days) vs LPS HAPI IV ONC (7 days)	Yes	P ≤ 0.001
LPS HAPI IV (14 days) vs ONC 4 days	No	ns
LPS HAPI IV (14 days) vs ONC 7 days	No	ns
LPS HAPI IV (14 days) vs ONC 14 days	Yes	P ≤ 0.001
LPS HAPI IV (14 days) vs control	Yes	P ≤ 0.001
LPS HAPI IV (14 days) vs LPS HAPI IV ONC (14 days)	Yes	P ≤ 0.001
ONC 4 days vs ONC 7 days	Yes	P ≤ 0.01
ONC 4 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 4 days vs control	Yes	P ≤ 0.001
ONC 4 days vs LPS HAPI IV ONC (4 days)	Yes	P ≤ 0.001
ONC 7 days vs ONC 14 days	Yes	P ≤ 0.001
ONC 7 days vs control	Yes	P ≤ 0.001
ONC 7 days vs LPS HAPI IV ONC (7 days)	Yes	P ≤ 0.001
ONC 14 days vs control	Yes	P ≤ 0.001
ONC 14 days vs LPS HAPI IV ONC (4 days)	No	ns
ONC 14 days vs LPS HAPI IV ONC (7 days)	No	ns
ONC 14 days vs LPS HAPI IV ONC (14 days)	No	ns
control vs LPS HAPI IV ONC (4 days)	Yes	P ≤ 0.001
control vs LPS HAPI IV ONC (7 days)	Yes	P ≤ 0.001
control vs LPS HAPI IV ONC (14 days)	Yes	P ≤ 0.001
LPS HAPI IV ONC (4 days) vs LPS HAPI IV ONC (7 days)	No	ns
LPS HAPI IV ONC (4 days) vs LPS HAPI IV ONC (14 days)	No	ns
LPS HAPI IV ONC (7 days) vs LPS HAPI IV ONC (14 days)	No	ns

Figure 7.9: Micrographs of whole eye cups and 12 µm frozen transverse sections of retinas 4 days after injection of LPS activated HAPI cells labelled with WGA-TR (red) into the vitreous. (A) Retina without ONC. The retina is inflamed as indicated by the black boundary. **(B)** Frozen transverse section of retina from (A) shows HAPI cell present in the vitreous. **(C)** Retina without ONC. This retina is the same condition at (A) but with no inflammation. **(D)** No HAPI cells are present. The retina from (C) and (D) has 3 times the RGCs than the retina from (A) and (B). **(E) and (F)** Retina with ONC. **(G)** Retina with ONC. There is lots of inflammation in the whole eye. Major parts of the retina are missing. (blue boundary indicates the amount of retina present). **(H)** No RGCs are present as labelled by Brn3a (green) but many HAPI cells are present (red with white arrows).


Eye Cup
Transverse Section
No Injury
Optic Nerve Crush
A
B
GCL
C
D
GCL
INL
ONL
E
F
GCL
INL
ONL
G
H
GCL
Sclera

CHAPTER 8: RETINAL GANGLION CELL SURVIVAL AFTER INJECTION OF MINOCYCLINE TREATED HAPI CELLS

8.1 Results

The purpose of this study was to examine the effect of microglial inhibition on RGC survival after optic nerve injury. Microglial inhibition was accomplished using minocycline, a well described modulator of neuroinflammation. Because minocycline may have effects on multiple cell types in the retina (such as retinal pigmented epithelium), we treated rat microglia *in vitro* with minocycline and injected those cells into rats with and without ONC. It was my hypothesis that inhibiting microglia from acquiring the M1 phenotype after injury will be neuroprotective, but dependent on the time course of the injury and where the microglia are present. An overview of this chapter is in Appendix V.

8.1.1 Migration of HAPI Cells

Injected HAOU cells were differentiated from other retinal neurons because they were labeled prior to injection. After minocycline treated HAPI cells were injected into the tail vein, no HAPI cells were present in the retina (figure 8.1) 4-14 days after injection, regardless of there being an injury or not. However, if there was an optic nerve crush injury (ONC) and the minocycline treated HAPI cells were injected into the tail vein, there were HAPI cells radiating from the crush site 4-14 days after injury (figure 8.2).

HAPI cells were not seen in the retina after minocycline treated HAPI cells were injected into the vitreous in the absence of an injury (figure 8.4). However, HAPI cells were first seen in the retina 7 days after injury. It was not

until 14 days after injection that many more HAPI cells were observed in the retina. The presence of numerous HAPI cells was often associated with inflammation, to the point that the retinal layers were not distinguishable (figure 8.4F). HAPI cells were not seen in the optic nerve after minocycline treated HAPI cells were injected into the vitreous whether there was an injury or not (figure 8.5).

8.1.2 RGC Loss after Optic Nerve Crush

Non-injured control retinas had 58.40 ± 4.71 RGCs/mm (n=7). Four days after ONC there were 44.90 ± 3.81 RGCs/mm (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$), this was a loss of 18.0 % of RGCs. At 7 days after ONC there was a loss of 37.7 % of RGCs with 34.12 ± 1.79 RGCs/mm left (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). After 14 days of ONC there was a loss of 65.4 % of RGCs with 18.9 ± 1.05 RGCs/mm remaining (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). These values were used as controls for experiments introducing minocycline treated HAPI cells into the vitreous or tail vein.

8.1.3 RGC Loss after Tail Vein HAPI Cell Injection

There was no significant loss of RGCs when minocycline treated HAPI cells were injected into the tail vein without injury (figure 8.3A). The RGC density 4 days after injection of minocycline treated HAPI cells without ONC was 58.77 ± 6.79 RGCs/mm (n=6; figure 8.3A). This was not significantly different from the control (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$) or from HAPI

cells injected into the tail vein with no treatment (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). Similarly, the RGC density 7 days after injection of minocycline treated HAPI cells into the tail vein was 59.19 ± 5.25 RGCs/mm (n=6) and 52.16 ± 6.31 RGCs/mm (n=6) 14 days after injection of minocycline treated HAPI cells. This was not significantly different from the control (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$) or when untreated HAPI cells were injected into the tail vein without injury (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$).

Injection of minocycline treated HAPI cells into the tail vein accompanied by an ONC resulted in greater RGC death than expected from ONC alone or when untreated HAPI cells were injected into the tail vein with ONC (figure 8.3B). After 4 days of injecting minocycline treated HAPI cells into the tail vein with ONC, there were 18.69 ± 7.27 RGCs/mm remaining (n=6). This was a loss of 58.4% of the RGCs compared to the loss expected from ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 58.1% less RGCs than when untreated HAPI cells were injected into the tail vein with ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$). There were 14.16 ± 5.58 RGCs/mm (n=6) remaining 7 days after minocycline treated HAPI cells were injected into the tail vein with ONC. This was 58.5% less RGCs than expected after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) and 48.3% less than the RGC density 7 days after untreated HAPI cells were injected into the tail vein with ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$). After 14 days of injecting minocycline treated HAPI cells into the tail vein and ONC, there was a

RGC density of 8.32 ± 1.74 RGCs/mm (n=6). This was 56.1% less than the RGC death expected after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$) but not significantly different than 14 days after untreated HAPI cells were injected into the tail vein with ONC (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$).

8.1.4 RGC Loss after Intravitreal HAPI Cell Injection

When minocycline treated HAPI cells were injected into the vitreous without ONC 4 days previously there was an RGC density of 61.38 ± 5.75 RGCs/mm (n=6; figure 8.6A). This was not statistically different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$) or 7 days after untreated HAPI cells were injected into the vitreous without ONC (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). There were 62.14 ± 5.76 RGCs/mm (n=6) 7 days after minocycline treated HAPI cells were injected into the vitreous without injury. This was not statistically different from controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$) or when untreated HAPI cells were injected into the vitreous (n=6; ANOVA/Tukey's post-hoc test). Similarly, 14 days after minocycline treated HAPI cells were injected into the vitreous there was an RGC density of 56.51 ± 4.73 RGCs/mm (n=6). This was statistically similar to controls (n=7; ANOVA/Tukey's post-hoc test; $p > 0.05$) but there was 23.2% more RGCs than when untreated HAPI cells were injected into the vitreous without injury (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$).

The RGC density 4 days after the injection of minocycline treated HAPI cells into the vitreous accompanied by an ONC was 26.37 ± 10.02 RGCs/mm (n=6; figure 8.6B). This was 41.3% more loss of RGCs than expected after ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.001$) but not statistically different from the RGC density when untreated HAPI cells are injected into the vitreous with ONC (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). Similarly, 7 days after minocycline treated HAPI cells were injected into the vitreous with ONC, there were 22.87 ± 8.64 RGCs/mm (n=6). This is 33.0% less than what was expected 7 days after ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.05$) and statistically similar to the density 7 days after untreated HAPI cells were injected into the vitreous with ONC (n=6; ANOVA/Tukey's post-hoc test; $p > 0.05$). After 14 days of injecting minocycline treated HAPI cells into the vitreous with ONC there were 8.25 ± 6.96 RGCs/mm (n=6). This represented a loss of 56.5% of RGCs compared to ONC alone (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$) and 54.8% compared to when untreated HAPI cells were injected into the vitreous with ONC (n=6; ANOVA/Tukey's post-hoc test, $p \leq 0.01$).

The large variability in RGC cell counts when minocycline treated HAPI cells were injected into the vitreous with ONC suggests that there were 2 conditions with high and low inflammation. When minocycline treated HAPI cells were injected into the vitreous with ONC 4 days earlier, some retinas appeared normal (figure 8.8A) and the eye cups show no sign of inflammation (figure 8.8C). Other eye cups had obviously high amounts of inflammation (figure

8.8D, blue boundary) and much of the retina had degenerated (figure 8.8B). When the retinal samples were sorted on these bases of high inflammation and low/no inflammation there was 2 trends in RGC survival that became apparent (figure 8.6C). When there were high levels of inflammation, the RGC densities were 0 RGCs/mm 4 days after injection (n=2), 0 RGCs/mm 7 days after injection (n=2), and 2.67 ± 3.2 RGCs/mm 14 days after injection (n=5). When there was low levels of inflammation the RGC density was 39.55 ± 4.85 RGCs/mm 4 days after injection (n=4), 34.30 ± 3.92 RGCs/mm 7 days after injection (n=4), and 36.15 ± 13.56 RGCs/mm 14 days after injection (n=1). These values were not statistically different from densities expected after ONC alone, except at 14 days where there were 91% more RGCs than what was expected after ONC alone (note that at 14 days in the low inflammation group there was only 1 sample).

8.2 Discussion

Currently, there is a lack of treatment options for neurodegenerative disorders and lowering IOP remains the main treatment for glaucoma. Since neuroinflammation has been implicated as an important mediator of neuronal death, it has become an attractive therapeutic target. Microglial activation is an important component of neuroinflammation but whether it is neuroprotective or cytotoxic is poorly understood. There is interest in anti-inflammatory compounds, such as minocycline, for treatment of neurodegenerative disorders. Minocycline is a broad-spectrum, second generation tetracycline antibiotic that easily crosses the BBB (Aronson, 1980; Yong et al., 2004). Minocycline modulates the immune

response by inhibiting the release of MMPs, NO, and cytokines (Stirling, Koochesfahani, Steeves, & Tetzlaff, 2005). It also acts to reduce proliferation and activation of resting microglial cells and inhibits microglial migration (Dommergues, Plaisant, Verney, & Gressens, 2003; Horvath, Nutile-McMenemy, Alkaitis, & DeLeo, 2008; Nutile-McMenemy, Elfenbein, & DeLeo, 2007). A recent study has shown that minocycline may only inhibit M1 microglia, shifting the microglial population to a M2 state (Kobayashi et al., 2013). This may be why HAPI cells were seen in the tail vein 4-14 days after injury regardless of whether or not they are treated with minocycline. When minocycline treated HAPI cells were injected into the vitreous, there were very few HAPI cells seen 7 days after injury and there was a large infiltration 14 days after ONC. This may suggest that minocycline does not inhibit the migration of M2 microglia and that inhibition by minocycline is no longer effective 14 days after treatment.

Minocycline is generally thought to be neuroprotective where it has shown to delay the mortality of mice with a mutated huntingtin gene and enhanced the survival of RGCs after axotomy (Baptiste et al., 2005; Chen et al., 2000). It may also protect substantia nigra neurons from toxicity by MPTP and 6-OHDA, which is used to induce experimental Parkinson's disease in mice, by modulating microgliosis (Blum et al., 2001; He, Appel, & Le, 2001; Lin et al., 2003). However, the protective effect of minocycline has been questioned, with reports that minocycline failed to be protective against MPTP in primate models where there was greater loss of striatal dopaminergic terminals and worse motor score

(Diguet et al., 2004; Yang et al., 2003). This study shows that the protective effects of minocycline modulated microglia depends on the location where they are injected and where they respond to the injury. There were 56-59% fewer RGCs than expected from ONC alone when minocycline treated HAPI cells were injected into the tail vein followed by an ONC injury. When the HAPI cells were injected into the tail vein with ONC, the cells migrated to the crush site in the optic nerve. This suggests that inhibiting M1 microglia from acting at the optic nerve resulted in a neurotoxic environment that leads to RGC death. This was similar to what has been described after glial scarring due to spinal cord injury, where the inhibitory extracellular matrix prevented microglia from acting on the axon (Bradbury & Carter, 2011; Rolls et al., 2004). Axonal growth and sprouting was facilitated by using chondroitinase ABC to digest the ECM and allow microglia to have access to the axon (Bradbury & Carter, 2011; Rolls et al., 2004). Therefore, microglia activation at the axon may be an important in the survival and recovery of injured neurons.

We report two outcomes after minocycline treated HAPI cells were injected into the vitreous with ONC: (1) a pro-inflammatory response where there was high amount of inflammation and (2) where there was little or no inflammation (figure 8.6C and figure 8.7). However, the condition where there was little or no inflammation dominated at the beginning of the injury (day 4 and 7), whereas the pro-inflammatory response dominated later in the injury (day 14). The RGC survival 4 and 7 days after injection was similar to what was observed

after ONC alone. There were more surviving RGCs 7 days after injury than the number of RGCs present after untreated HAPI cells were injected into the vitreous with ONC (figure 8.6D). It was expected that the RGC survival would be the same as that after ONC because the resident microglia and other cells have not been affected by minocycline. Therefore, any RGC survival more than what would be expected after untreated HAPI cells are injected with ONC could be considered protective. These results showed that there were conditions where minocycline treatment was harmful after injury, however, inhibition of microglia was beneficial to the survival of neurons when the microglia response was associated with the cell body. This suggests that how the minocycline is administered and where microglia are activated/inhibited is an important factor that determines its effect on the survival of neurons. Activated and resting microglia are found in grey and white matter of the brain but the proportion of activated microglia becomes greater in white matter during aging (Ogura, Ogawa, & Yoshida, 1994). This finding may explain age related degenerative changes in the brain. In addition, after middle cerebral artery occlusion there were more ramified and resting microglia in the subventricular zone and more activated microglia in the striatum (Thored et al., 2008). Therefore, different anatomical localizations affect how microglia are activated differently. Furthermore, microglia can be neuroprotective or toxic dependent on where they act in the injury.

Minocycline may act by suppressing microglia polarization to the M1 state early in injury but it has shown to not be able to completely inhibit the expression of M1 state (Kobayashi et al., 2013). This may explain why in some cases there was a pro-inflammatory response in retinas that had minocycline treated HAPI cells injected into the vitreous with ONC. Minocycline is also not able to enhance the expression of M2 phenotype, but just inhibits the M1 phenotype which shifts the microglial population to be predominantly of the M2 type (Kobayashi et al., 2013). Minocycline also only partially suppresses the production of cytokines such as IL-6, TNFα, and IL 1β by inhibiting the nuclear translocation of NF-κB (Kobayashi et al., 2013; Pang, Wang, Benicky, & Saavedra, 2012). Long term treatment with minocycline has shown to be safe and has minor side effects (Bonelli, Heuberger, & Reisecker, 2003). In addition, this study shows that minocycline treated HAPI cells injected into the tail vein or vitreous resulted in no loss of RGCs when there was no injury. So minocycline treated microglia do not produce the same cytotoxicity that was seen when LPS activated HAPI cells were injected into the vitreous (Chapter 7).

Microglial inhibition and treatment with minocycline may have great therapeutic potential early in neurodegenerative disorders or CNS injury. In glaucoma, lowering IOP decreases the progression of RGC death but does not stop it (Bosco et al., 2008). Due to the multifaceted nature of glaucoma, targeting multiple pathways involved in the degeneration of RGCs may be the best solution to slowing the progress of neurodegeneration or may be even halting it. This study

provides valuable information on how minocycline and different microglial activation states affects survival of RGCs. Inhibition of microglia is only beneficial when it is inhibiting the microglia by the cell body and not the axon after injury (figure 8.9). In addition, the benefit is only seen early in the injury. Once the injury has become too severe and perhaps the microglial inhibition is lifted, the outcome is detrimental. Long term treatment of DBA/2J glaucoma mice with minocycline before any clinical signs of glaucoma was shown to suppress microglial activation and improve RGC survival (Bosco et al., 2008). Minocycline was neuroprotective and reduced the volume of injured neurons 24 hours, but not 7 days, after middle cerebral artery occlusion and reperfusion (Fox et al., 2005). In ALS, minocycline treatment after the onset of the disease in mice did not prolong their survival but was beneficial presymptomatically (Keller, Gravel, & Kriz, 2011). This suggests that early inhibition of microglia early after CNS injury is an ideal therapeutic window. However, more research is needed to elucidate the mechanisms whereby minocycline causes its inhibition and neuroprotection. Other more complete inhibitors of microglia should be investigated for their ability to affect survival of neurons after injury or neurodegeneration.

8.3 Figures and Tables

Figure 8.1: 12 µm thick frozen traverse section of rat retina after tail vein injection of minocycline treated HAPI cells for 4-14 days. (A) 4 days after injection of the cells into the tail vein, there were no HAPI cells (red) present in the retina or vitreous. **(B)** Similarly, 4 days after injection and ONC of cells into the tail vein, there were no HAPI cells present in the retina. **(C)** 7 days after injection of the cells into the tail vein, there were no HAPI cells present in the retina or vitreous. **(D)** This trend continues 7 days after injection of cells into the tail vein and ONC where there were no HAPI cells present in the retina. **(E) and (F)** Similar to the other time points, there were no HAPI cells in the retina 14 days after injection with or without ONC. The retinal ganglion cells were labeled with Brn3a (green).

GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

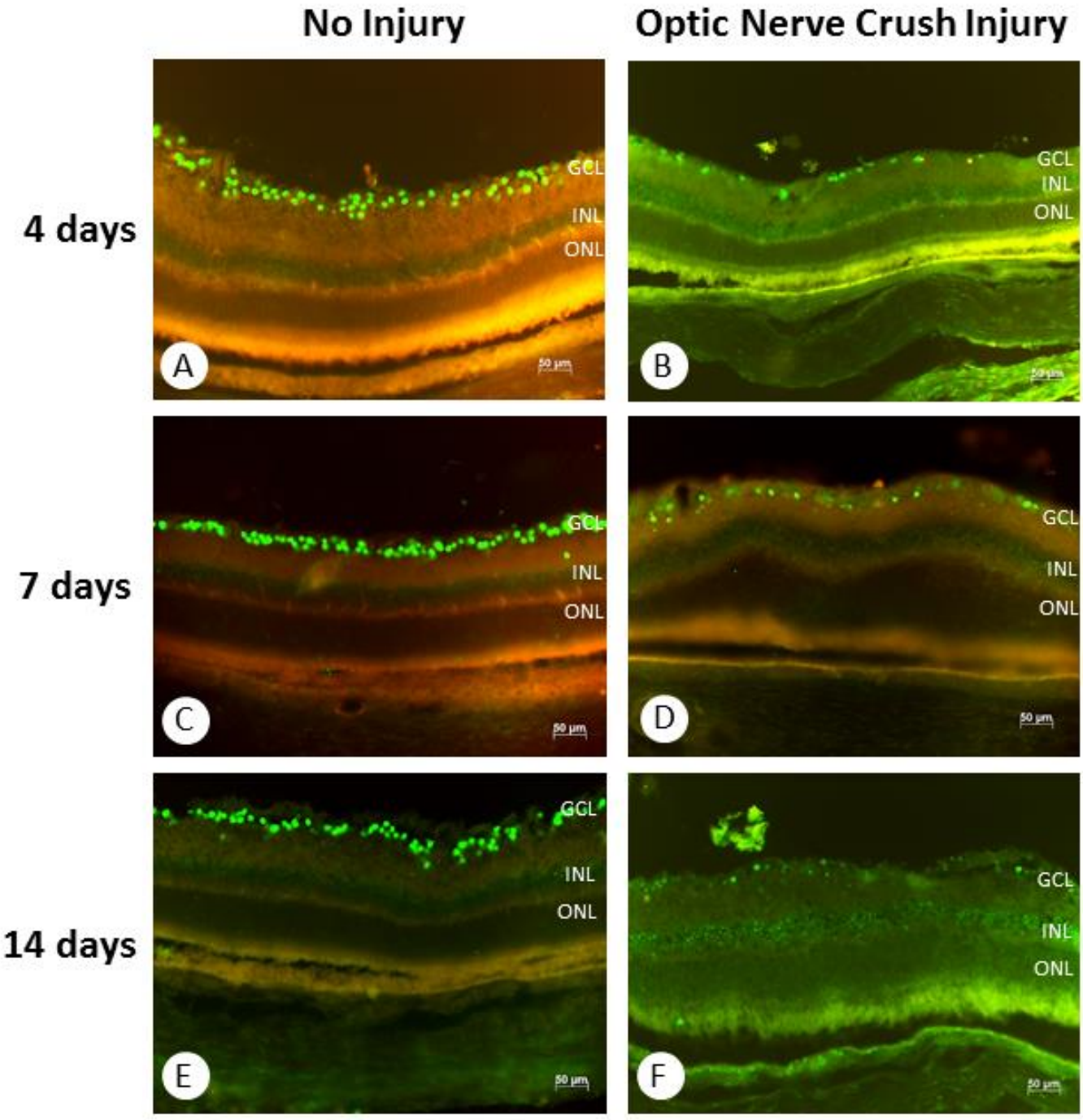

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
GCL
INL
ONL
A
B
C
D
E
F
50 µm

Figure 8.2: 12 µm thick frozen sections of optic nerve after injection of minocycline treated HAPI cells (red) injected into the tail vein. (A) 4 days after injection but without ONC there were no HAPI cells present in the optic nerve. **(B)** 4 days after injection and with ONC there were HAPI cells present in the optic nerve at the crush site. **(C)** 7 days after injection but without ONC there were no HAPI cells present in the optic nerve. **(D)** 7 days after injection and with ONC there were HAPI cells in the optic nerve at the crush site radiating outward. (**E)** 14 days after injection but in the absence of ONC there were no HAPI cells in the optic nerve. **(F)** 14 days after injection and with ONC there were HAPI cells in the optic nerve at the crush site.

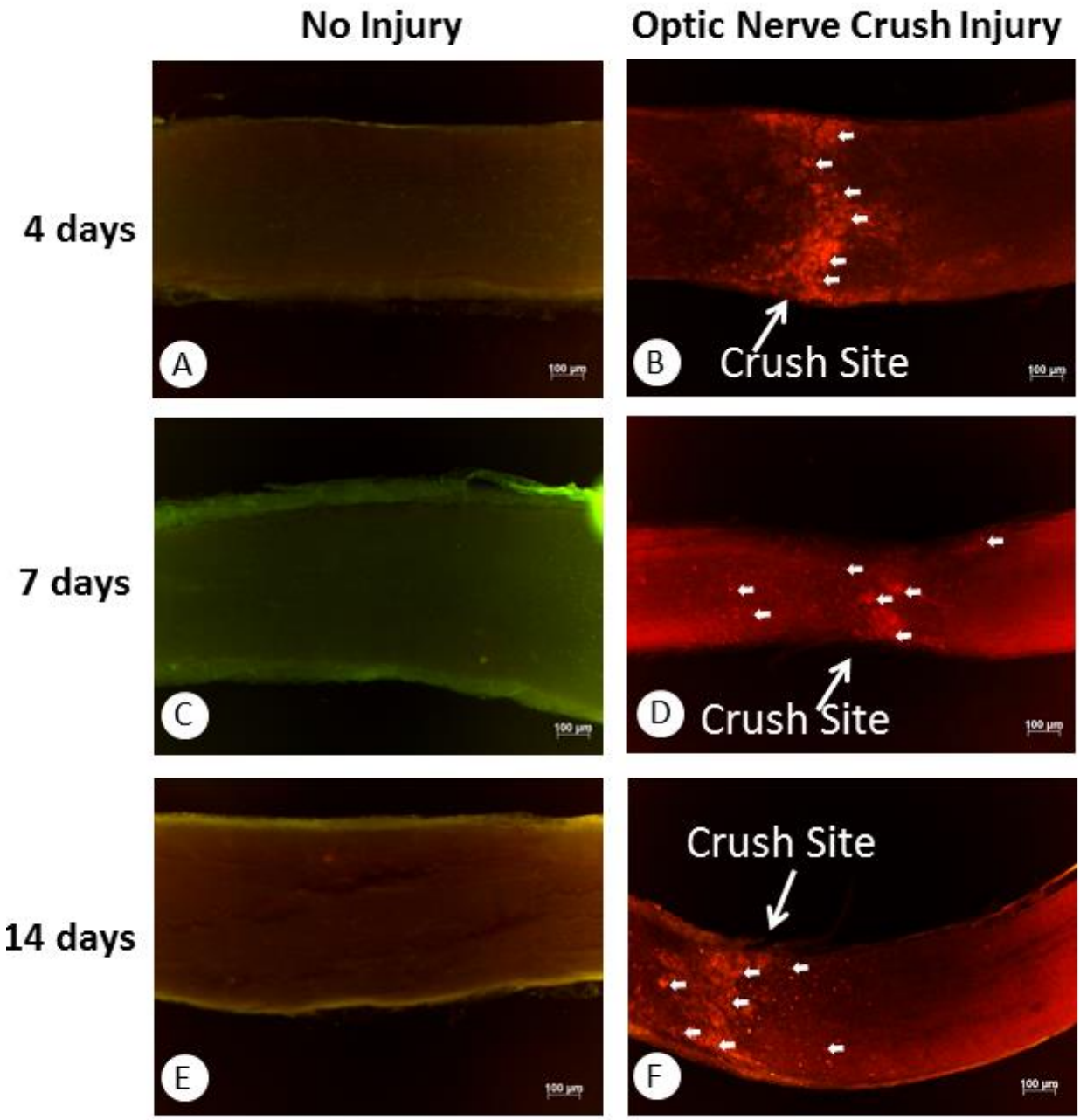
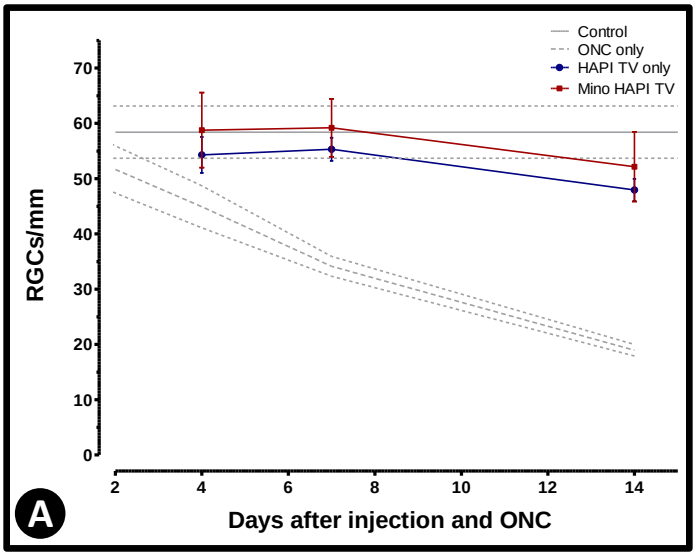
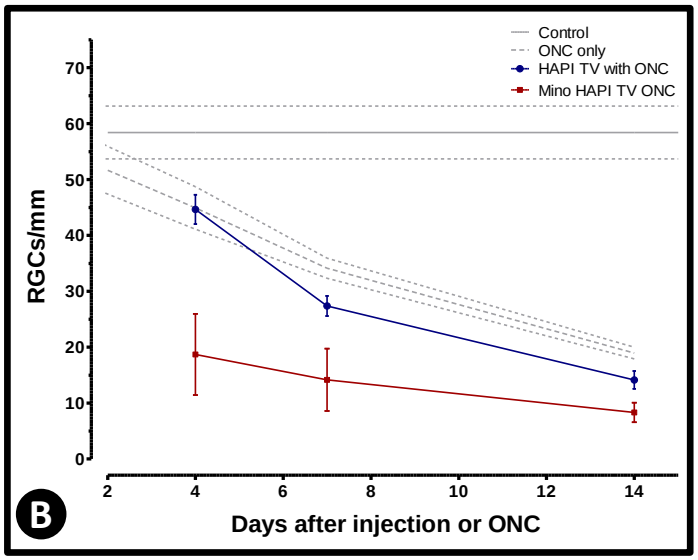

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
A
B
Crush Site
C
D
Crush Site
E
F
Crush Site
100 µm

Figure 8.3: The RGC density 4-14 days after injection of minocycline treated HAPI cells into the rat tail vein. (A) The RGC density was not different from the control (grey solid line) or from when untreated HAPI cells were injected into the tail vein without injury (HAPI TV; blue circle) when minocycline treated HAPI cells were injected into the tail vein without ONC (Mino HAPI TV; red square). **(B)** When minocycline treated HAPI cells were injected into the tail vein with ONC (Mino HAPI TV ONC; red square), the RGC loss was greater than what was expected after ONC alone (grey dotted line) or when untreated HAPI cells were injected into the tail vein with ONC (HAPI TV ONC; blue circles). **(C)** Injection of minocycline treated HAPI cells into the tail vein with ONC (blue circle) was cytotoxic to RGC, however, when the cells were injected without ONC (red square) there was no effect on RGC survival.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


Control
ONC only
HAPI TV only
Mino HAPI TV
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after injection and ONC
A


Control
ONC only
HAPI TV with ONC
Mino HAPI TV ONC
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after injection or ONC
B

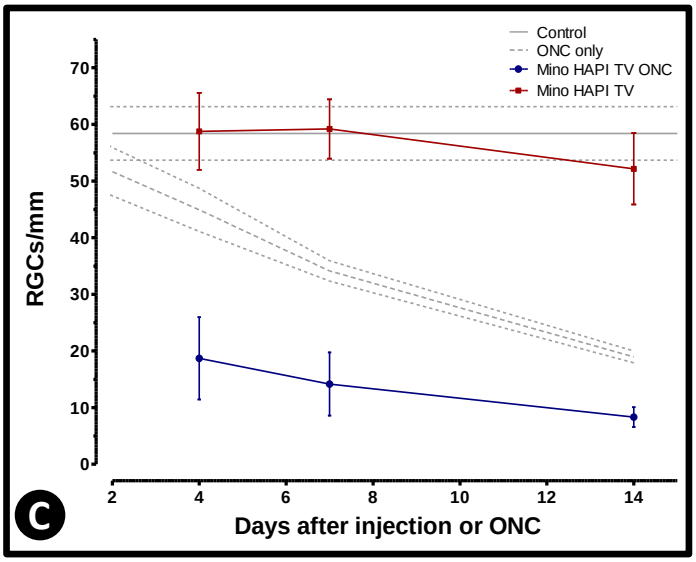

Control
ONC only
Mino HAPI TV ONC
Mino HAPI TV
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after injection or ONC
C

Figure 8.4: 12 µm thick frozen traverse section of rat retina after intravitreal injection of minocycline treated HAPI cells for 4-14 days. (A) 4 days after injection of the cells there were no HAPI cells (red) present in the retina or vitreous. **(B)** Similarly, 4 days after injection and ONC there were no HAPI cells present in the retina. **(C)** 7 days after injection of the cells there were no HAPI cells present in the retina or vitreous. **(D)** 7 days after injection and ONC there were a small number of HAPI cells present in the retina (white arrows). **(E)** 4 days after injection of the cells there were no HAPI cells present in the retina or vitreous. **(F)** 14 days after injection with ONC the retina was very inflamed that even the retina layers were not distinguishable. There were many HAPI cells and may be other auto-fluorescent immune cells present in the retina. The retinal ganglion cells were labeled with Brn3a (green).

GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

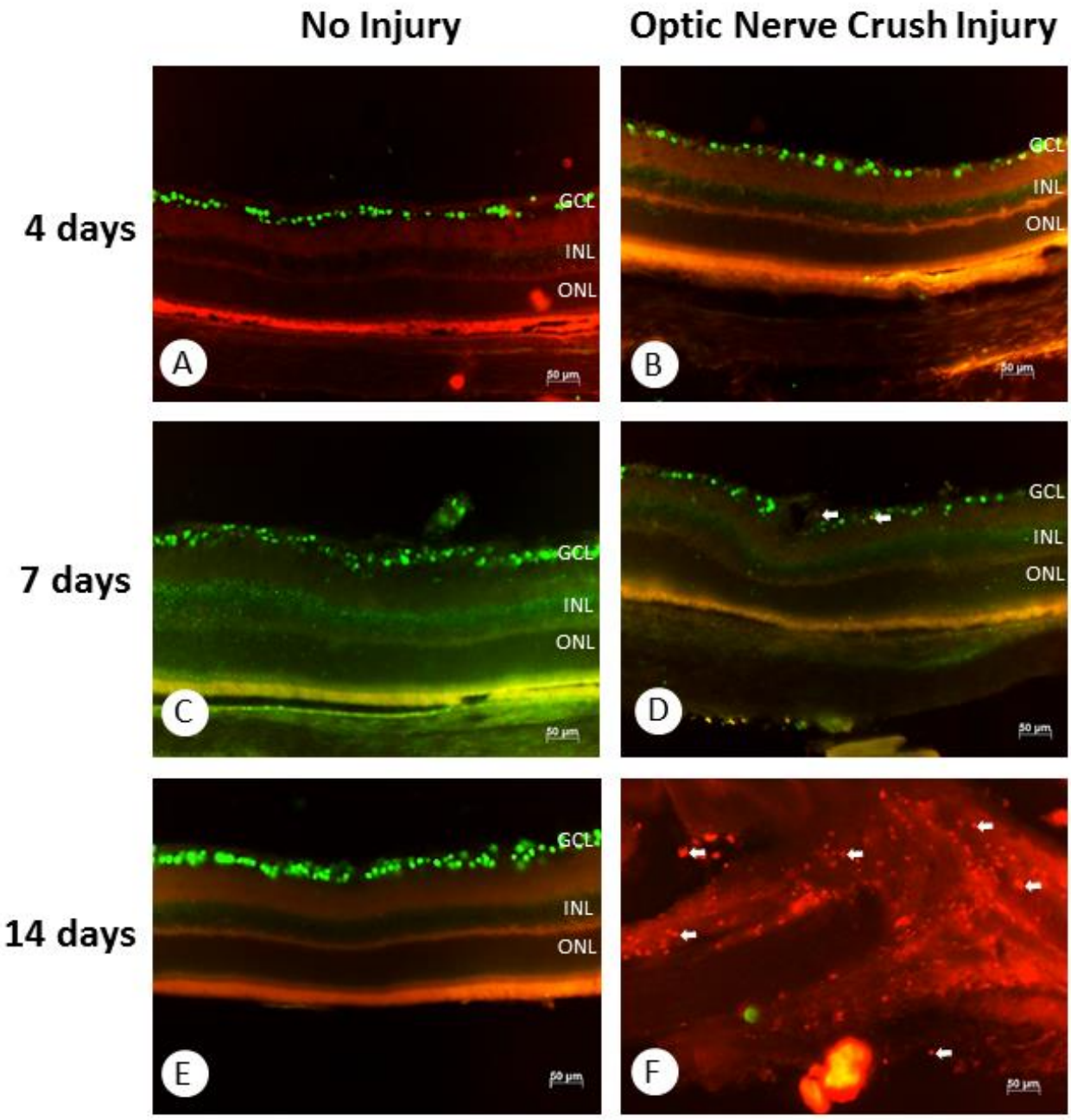

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
GCL
INL
ONL
A
B
C
D
E
F
50 µm

Figure 8.5: 12 µm thick frozen sections of optic nerve after injection of minocycline treated HAPI cells (red) injected into the vitreous. There were no HAPI cells in the optic nerve 4-14 days after the injection of minocycline treated HAPI cells whether there was no injury (A, C, E) or optic nerve crush injury (B, D, F).

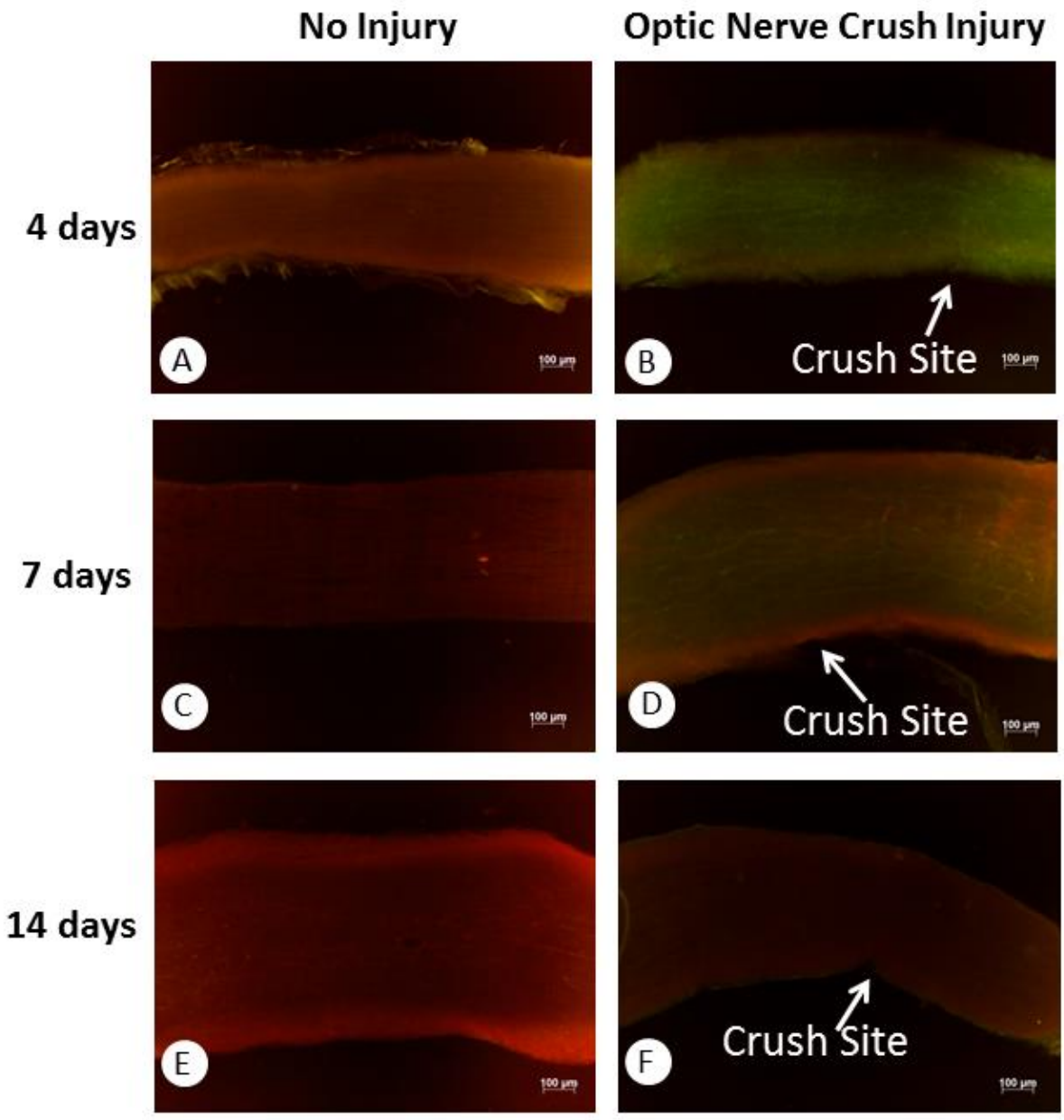

No Injury
Optic Nerve Crush Injury
4 days
7 days
14 days
A
B
C
D
E
F
Crush Site
Crush Site
Crush Site
100 µm

Figure 8.6: The RGC survival 4-14 days after intravitreal injection of minocycline treated HAPI cells. **(A)** The RGC density was not different from controls (grey line) when minocycline treated HAPI cells were injected into the vitreous without ONC (red squares). The RGC density did not differ from when untreated HAPI cells were injected into the vitreous without ONC (blue circles) at 4 and 7 days, however, the RGC density was greater than the RGC density 14 days after untreated HAPI cells were injected into the tail vein without ONC. **(B)** When the RGC densities after injection of minocycline treated HAPI cells and ONC (red squares) were pooled, it was not different from when untreated HAPI cells were injected into the vitreous and there was an ONC (blue circles). This would suggest that minocycline treated microglia are not neuroprotective after injury. **(C)** However, it is possible that there are two groups that determine RGC survival differently due to the large confidence intervals (red squares). When the retinas were split into a high inflammation (green circles) and a low/no inflammation (black circles) group, there was a greater loss of RGCs in the high inflammation group and greater survival in the low/no inflammation group. The RGC density in the low inflammation group was not different from the RGC density expected after ONC (grey dotted line). **(D)** The data points with the greatest sample size were taken from the two groups (4 days: n=4, low inflammation; 7 days: n=4, low inflammation; 14 days: n=5, high inflammation) to determine the dominate trend. Minocycline treated HAPI cells may be neuroprotective early in the injury after intravitreal injection and cytotoxic later in

the injury. Since the indigenous microglia were not inhibited, we would still expect the loss that is seen after ONC. The RGC density was not different from the density 4 days after untreated HAPI cells were injected into the vitreous with ONC. The RGC survival was greater at 7 days after injury but there was greater RGC loss 14 days after injury.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

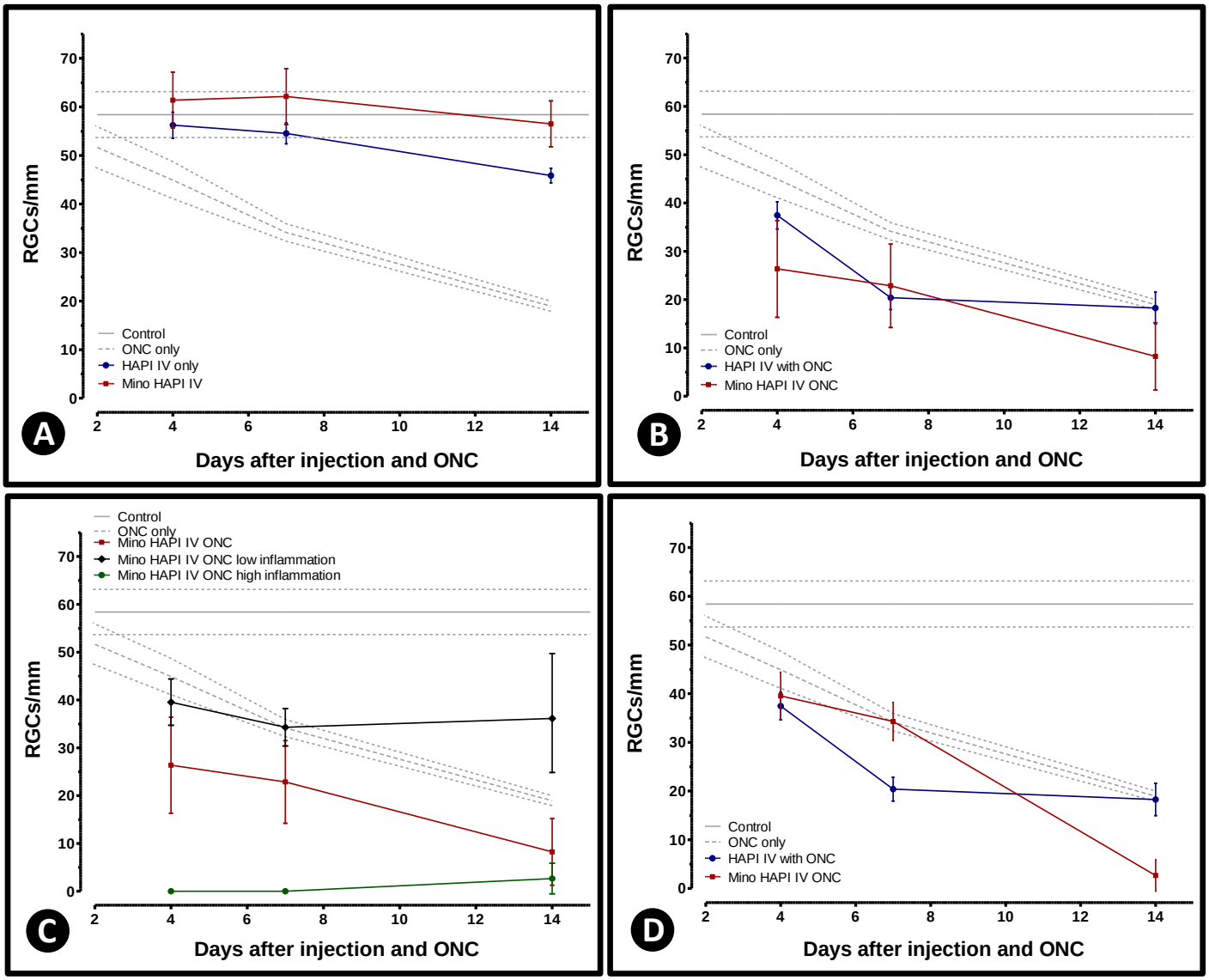

A
RGCs/mm
Days after injection and ONC
Control
ONC only
HAPI IV only
Mino HAPI IV
B
RGCs/mm
Days after injection and ONC
Control
ONC only
HAPI IV with ONC
Mino HAPI IV ONC
C
RGCs/mm
Days after injection and ONC
Control
ONC only
Mino HAPI IV ONC
Mino HAPI IV ONC low inflammation
Mino HAPI IV ONC high inflammation
D
RGCs/mm
Days after injection and ONC
Control
ONC only
HAPI IV with ONC
Mino HAPI IV ONC

Figure 8.7: The RGC survival trend after injection of minocycline treated HAPI cells into the vitreous. The RGC density did not change from values for the controls (grey solid line) when minocycline treated HAPI cells were injected without ONC (red square). There was a high inflammation and low/no inflammation group when minocycline treated HAPI cells were injected into the vitreous with ONC. The values with the greatest sample size from the two groups were taken to determine the trend for the RGC survival after injection of minocycline treated HAPI cells into the vitreous with ONC (blue circles). The RGC density 4 and 7 days after injection were not different than the values expected after ONC alone (grey dotted line). However, 14 days after there were much fewer surviving RGCs than there would be after ONC alone at the same time point.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

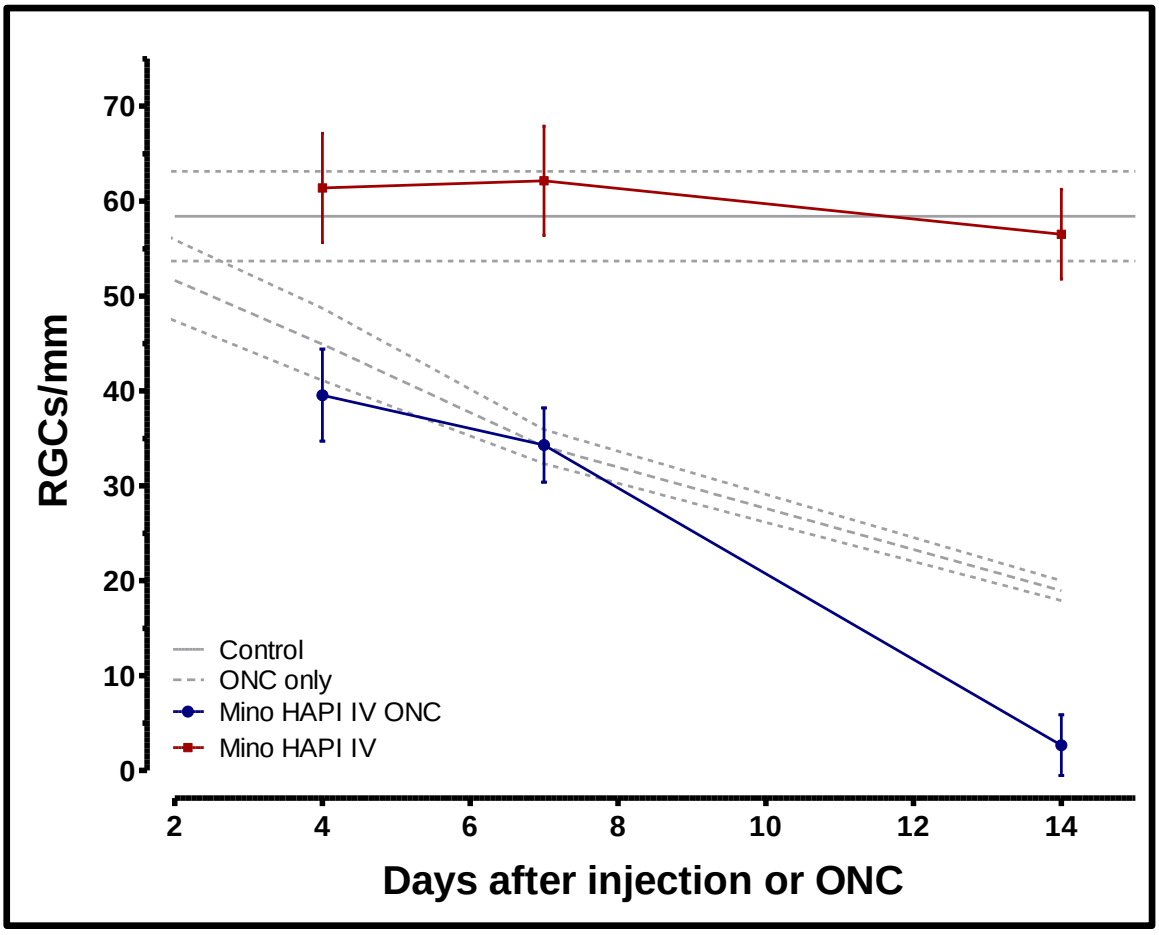

RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after injection or ONC
Control
ONC only
Mino HAPI IV ONC
Mino HAPI IV

Figure 8.8: High and low/no inflammation of retinas after minocycline treated HAPI cells were injected into the vitreous with optic nerve crush. RGC density can be categorized by a high inflammation and low/no inflammation group. When there was low/no inflammation **(A)** the retina shows almost no HAPI cells or immune infiltrate. **(B)** The eye cup of retinas with low/no inflammation did not show any swelling and the eye cup looked normal. **(C)** However, when the retinas were highly inflamed, it was hard to distinguish the retinal layers and very little RGCs (green) were present. **(D)** The eye cup also had a large area of inflammation indicated by swelling or puffiness (blue boundary). In some cases the retina were missing or very thin.

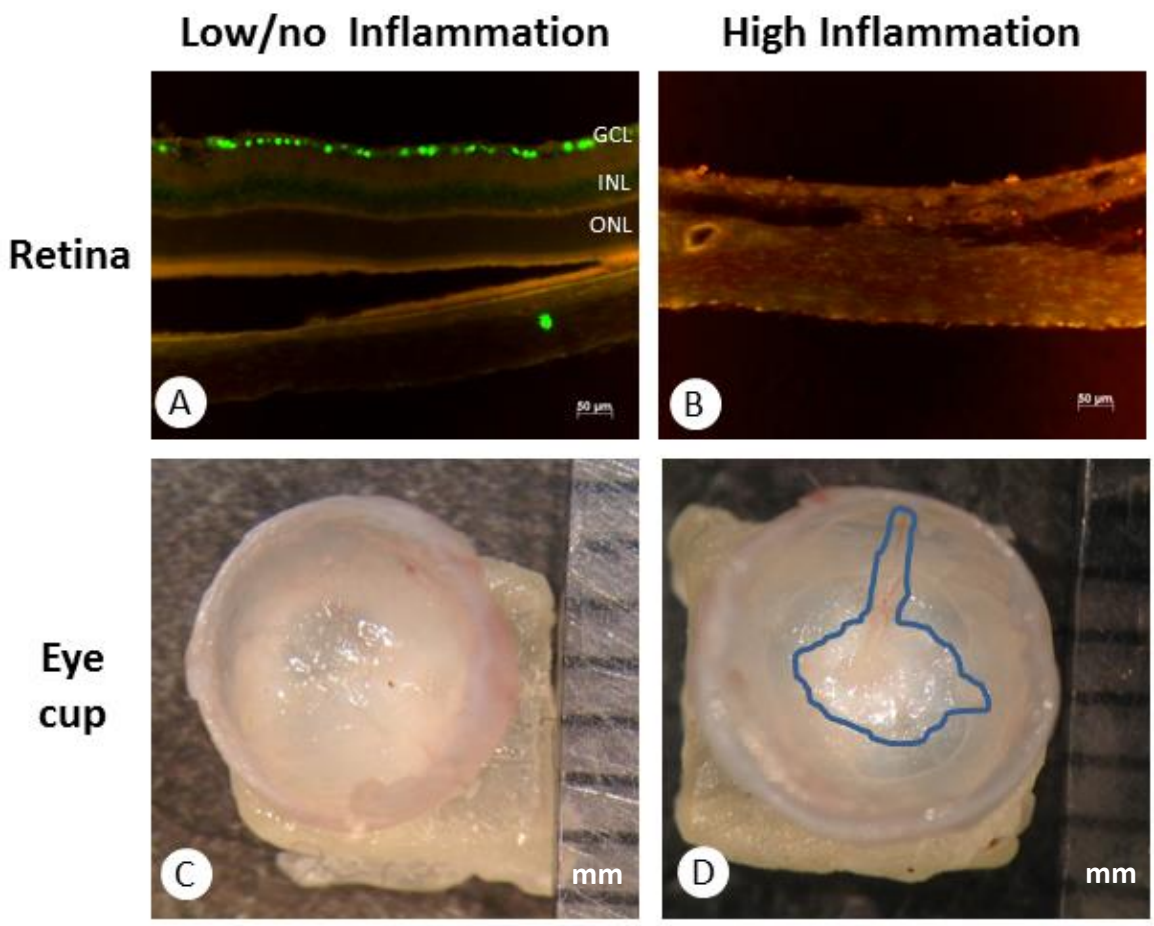
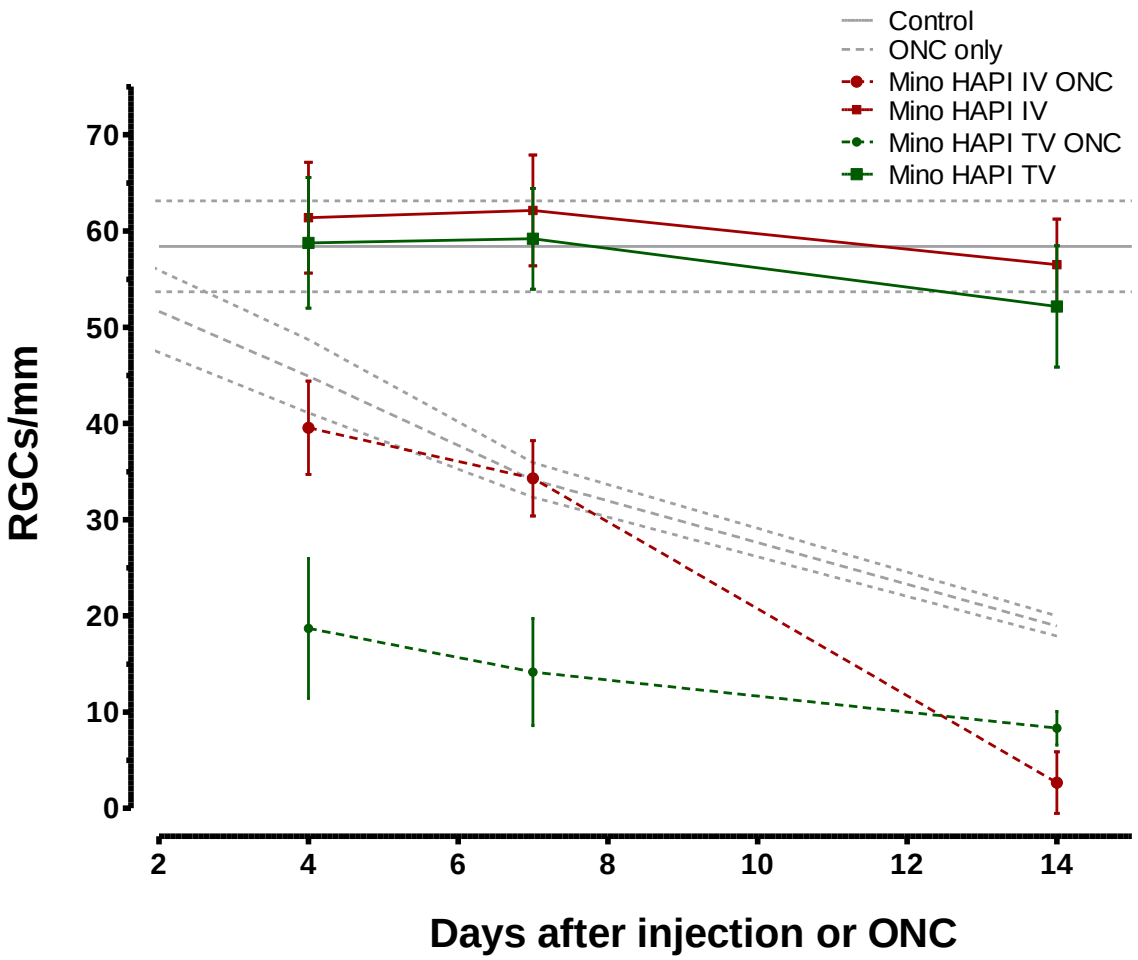

Low/no Inflammation
High Inflammation
Retina
Eye cup
GCL
INL
ONL
A
B
C
D
mm
mm

Figure 8.9: RGC survival 4-14 days after minocycline treated HAPI cells were injected into the vitreous or tail vein with or without optic nerve crush. There was no significant loss of RGCs when minocycline activated HAPI cells were injected into the vitreous (red square) or tail vein (green square) without injury to the optic nerve compared to the control (grey solid line; dotted lines are the confidence interval). Minocycline treated HAPI cells were neuroprotective early in the injury when they were injected into the vitreous (red circles) and there was an ONC because there was no additional loss of RGCs when compared to the ONC alone (grey dotted line). However, later in the injury (14 days), the minocycline treated HAPI cells were no longer neuroprotective when injected into the vitreous and resulted in greater death than expected from ONC alone. There was greater RGC loss when injected into the tail vein with ONC (green circle) than that would be expected from the ONC alone. This suggests that microglial inhibition at the optic nerve is cytotoxic.

Error bars = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


Control
ONC only
Mino HAPI IV ONC
Mino HAPI IV
Mino HAPI TV ONC
Mino HAPI TV
RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after injection or ONC

CHAPTER 9: CONCLUSION

9.1 A Neuroinflammatory Role in Glaucoma

Glaucoma is the second leading cause of blindness in the world and is characterized by damage to the optic nerve that leads to slow progressive death of RGCs (Quigley & Broman, 2006). The dominate theory for the pathology of glaucoma is that changes after raised intraocular pressure (IOP) impinges the optic nerve that blocks the transport of neurotrophic factors back to the soma initiating the apoptosis of the RGCs (Almasieh, Wilson, Morquette, Cueva Vargas, & Di Polo, 2011). However, glaucoma can occur without high IOP and lowering IOP does not prevent the loss of RGCs (Ishida, Yamamoto, & Kitazawa, 1998). Some other factors that have now become associated with glaucoma are glial activation, release of tumor necrosis factor (TNF), oxidative stress, mitochondrial dysfunction, and dysregulation of the immune system (Ju et al., 2008; Schori et al., 2001; G. Tezel, 2006; G Tezel & Wax, 2000, 2003). It is clear that there is a need for multifaceted treatments in glaucoma but decreasing IOP is the main therapeutic intervention currently available.

9.2 Neuroinflammatory Mechanism of Neuronal Death

Inflammation in neurodegenerative disorders, like glaucoma, is starting to get more attention in the last 20 years. It used to be thought that glial cells (microglia, astrocytes, and oligodendrocytes) just played a supportive role in the CNS, in fact the word glia is derived from the Greek word for glue (Allen & Barres, 2009). Post-mortem analysis of brains showed that many inflammatory mediators, such as complement, cytokines, chemokines, and acute phase proteins,

are involved in the brain regions associated with neurodegenerative disorders (Akiyama et al., 2000). Glial cells are normally beneficial to the development and maintenance of the CNS, however, sometimes they become over activated in a chronic neuroinflammatory cycle that can result in, or exacerbate, neurodegenerative disorders (Griffin et al., 1998). It is the balance between those states that is the focus of recent studies in neuroinflammation. Glial activation has become an attractive target for therapeutic interventions to treat neurodegenerative disorders (Craft, Watterson, & Van Eldik, 2005). Minocycline is being used for its anti-inflammatory properties and is being investigated as a treatment option for neurodegenerative disorders and CNS injury such as ischemia, traumatic brain injury, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, and multiple sclerosis (MS) (Chen et al., 2000; Choi et al., 2007; Jackson-Lewis et al., 2002; Luccarini et al., 2008; Mejia, Ona, Li, & Friedlander, 2001; Yong et al., 2004; Yrjänheikki et al., 1999; S. Zhu et al., 2002). Minocycline is able to modulate microglia by inhibiting cytokine production, microglia migration, matrix metalloproteases (MMPs), and nitric oxides (Stirling, Koochesfahani, Steeves, & Tetzlaff, 2005; Wasserman & Schlichter, 2007).

Microglia are the innate immune cell of the CNS and are key players in the neuroinflammatory response after CNS injury (Cuadros & Navascués, 1998). The normal function of microglial cells are to scavenge toxic agents, supply growth factors, and supply metabolites (G Tezel & Wax, 2003). In the retina,

microglial cells reside at the margins of the inner plexiform layer and in response to injury proliferate and migrate to the affected areas (Bodeutsch & Thanos, 2000; Santos et al., 2010). Activated microglial cells undergo phenotypic changes and release factors that have neurotrophic or cytotoxic action (Langmann, 2007).

Microglial acquire activation states on a spectrum between the classically activated (M1) phenotype or the alternatively activated (M2) phenotype (C.A. Colton et al., 2006; Mantovani et al., 2004). The M2 microglia are less inflammatory, characterized by a reduction in NO production and increase in anti-inflammatory cytokines (Lucin & Wyss-Coray, 2009). The outcome of microglial activation may be dependent on whether the response is too aggressive or too passive (Lucin & Wyss-Coray, 2009). A recent study demonstrated that minocycline may selectively inhibit M1 microglia and have no effect on M2 microglia (Kobayashi et al., 2013). Experimentally, lipopolysaccharides (LPS) have been used to elicit a strong inflammatory response and push the microglia into the M1 state (Hanisch & Kettenmann, 2007; Jang et al., 2007; Martinez, Sica, Mantovani, & Locati, 2008; Zheng et al., 2012).

Activated microglia are neurotoxic because they increase the oxidative stress of the tissue by over producing ROS and NO (Carter & Dick, 2003; Streit, 1993). The increased levels of nitric oxide synthase (NOS) results in the breakdown of the blood retinal barrier and increases leukostasis (Leal et al., 2007). Another way activated microglia may recruit immune cells to the CNS is by producing monocyte chemoattractant protein 1 (MCP-1) (D'Mello, Le, &

Swain, 2009). Some suggest that inhibition of microglial activation by minocycline can slow degeneration of retinal neurons in axotomized eyes, however, contradictory studies show that microglia activation after the onset of many neurological pathologies can be beneficial due to their ability to remove toxic cellular debris and secrete neurotrophic factors (Nakajima et al., 2001). Microglia derived NGF, BDNF, and CNTF may indirectly influence neuronal survival in the eye by modulating neurotrophin production by Müller cells (Harada et al., 2002).

9.3 Controversy and Problems with Studying Microglial Activation

The neuroprotective or pro-inflammatory role of microglia after injury is controversial. The function of over activated microglia or hypo-activated microglia after neuronal injury is not well understood. The nature and time course of the injury may determine what phenotype microglia acquire and determine the outcome of the activation. It was my hypothesis that manipulating the activation of microglia to acquire a pro-inflammatory or pro-survival phenotype would exacerbate neuronal cell death or enhance neuronal survival after injury, respectively. A major issue with studying microglial activation *in vivo* has been that many of the substances that are used to activate or inhibit microglia (such as LPS and minocycline) also have an action on other cell types in the CNS. LPS is known to activate astrocytes and Müller cells, therefore, the effect of LPS on neuronal survival could not be attributed to the microglia alone (Fernandes, Silva, Falcão, Brito, & Brites, 2004; Goureau, Hicks, Courtois, & De Kozak, 2002; Jang

et al., 2007; Schumann et al., 1998). Similarly, minocycline is known to affect retinal pigmented epithelium cells directly (Hollborn, Wiedemann, Bringmann, & Kohen, 2010).

9.4 A Method to Test Microglial Activation without Activated Other Cells

I wanted to determine if HAPI microglial cells, cultured *in vitro,* could be grafted into the retina or injected into the tail vein and migrate to the site of injury. This technique could be later used to activate the HAPI cells with different substances to alter their phenotype *in* vitro and then inject the cells back into the animal to see the effect of microglial activation with the substance without risking the activation of other cell types.

9.4.1 Migration of HAPI cells to the Retina and Optic Nerve

Untreated HAPI cells migrate to the retina or optic nerve only after optic nerve injury. As with resident microglia, an injury signal was required to activate the HAPI cells and to initiate migration to the injury site. The HAPI cells were seen at the injury site in the optic nerve after tail vein injection but were not present in the retina. When HAPI cells were injected into the vitreous, then the HAPI cells were seen in the retina and not in the optic nerve. Similar results were seen after minocycline treated HAPI cells were injected into the vitreous or tail vein. However, the response seemed delayed since there were very few HAPI cells in the retina 7 days after minocycline treated HAPI cells were injected into the vitreous. When HAPI cells were hyper-activated with LPS and injected into the vitreous, they migrated to the retina in the absence of an injury signal. In

addition, after LPS activated HAPI cells were injected into the vitreous with ONC, HAPI cells and/or other auto-florescent immune cells were seen in the optic nerve at the crush site. This suggests that LPS activated HAPI cells release factors that attract immune cells.

9.5 The Effect of Different Microglial Activation States on RGC Survival

After we had developed a novel technique for allografting HAPI cells that were successfully able to migrate to the injury site, we used different drugs and substances to activate HAPI cells without causing toxicity to the animal or activating other cell types. This method can help explore the dualistic nature of microglia after injury and helped determine what conditions were required to put HAPI cells into a neuroprotective or toxic phenotype.

9.5.1 How Different Activation States Effect RGC survival after Intravitreal Injection of HAPI cells

When untreated HAPI cells were injected into the vitreous without injury there was no loss of RGCs until 14 days after injection where there was a loss of 21% of the RGCs (Figure 9.1). This may be due to an increase in oxidative stress result from an increase in metabolism due to the addition of cells into the eye. This RGC loss was not seen when HAPI cells were treated with minocycline. However, when LPS activated HAPI cells were injected into the vitreous without optic nerve crush (ONC) there was a loss of RGCs comparable to that seen 7 days after ONC. Therefore, in the absence of injury, minocycline treated HAPI cells

were neuroprotective, whereas LPS activated HAPI cells were neurotoxic and resulted in a pro-inflammatory response.

When untreated HAPI cells were injected into the vitreous followed by an ONC injury there was a loss of RGCs at 4 and 7 days (figure 9.2). This loss was greater than would be expected after ONC alone. This additional loss was not seen 4 and 7 days after minocycline treatment of HAPI cells. However, later in the injury (14 days) there was a loss of RGCs greater than what was expected after ONC. LPS activation of HAPI cells after ONC also resulted in a pro-inflammatory response 4 days after injury and injection and was sustained over the 14 day period. Hypo-activation of HAPI cells by minocycline treatment resulted in RGC neuroprotection early in the injury but became pro-inflammatory later. Hyper-activated HAPI cells were pro-inflammatory and resulted in the ultimate death of RGCs.

9.5.2 How Different Activation States Effect RGC survival after Tail Vein Injection of HAPI cells

HAPI cells injected into the tail vein get introduced to the systemic system. There they can interact with the peripheral immune system and after injury will migrate to the optic nerve. This is different from injection into the relatively isolated vitreous where the HAPI cells interact with the cell body of the RGCs, instead of the axon. When untreated HAPI cells were injected into the tail vein without ONC there was no loss of RGCs until 14 days after injection (figure 9.3). This loss was similar to what was observed after intravitreal injection. This loss

was not seen after minocycline treatment or LPS activation. This may suggest that factors released by minocycline treated HAPI cells or LPS activated HAPI cells may be neuroprotective in the absence of injury either by themselves, or through immune-CNS communication.

There was a loss of RGCs later in the injury when HAPI cells were injected into the tail vein with ONC (figure 9.4). The opposite neuroprotective effect was seen after minocycline or LPS treated HAPI cells were injected into the tail vein with ONC than when HAPI cells were injected into the vitreous with ONC. After 4 days following injection of LPS activated HAPI cells into the tail vein with ONC there were as many RGCs present as in the control retinas. Whereas 4 days after HAPI cells were injected into the tail vein, there were 58% less RGCs than expected after ONC and this was sustained over the 14 day period. However, 7 days after injection of LPS activated HAPI cells into the tail vein with ONC there was a loss of RGCs similar to that seen after minocycline treated HAPI cells were injected. This shows that hyper-activated HAPI cells in the optic nerve are neuroprotective early in the injury, but result in a pro-inflammatory response later in the injury that leads to the loss of RGCs. Minocycline hypo-activated HAPI cells in the optic nerve after injury were cytotoxic and resulted in the loss of RGCs.

9.5.3 Differential Benefits of M1 and M2 Microglia

Microglia treated with minocycline were neuroprotective when acting on the cell body of the RGC early in the injury but not when acting on the axon. It is

possible that minocycline inhibited all microglia regardless of phenotype or selectively inhibit M1 microglia. The opposite is true for hyper activated (M1) HAPI cells where they are neuroprotective early in the injury by acting at the axon but not when at acting on the cell body. This may help explain the different effects of minocycline or hyper activated microglial cells in the literature. However, one thing is clear in these studies: long term activation or inhibition of microglia ultimately results in a pro-inflammatory response that exacerbates the loss of neurons. This reveals that any therapeutic intervention targeting microglia to treat neurodegeneration or CNS injury should be early in the injury and targeted to a specific location.

Microglia activated with high doses of LPS has been shown to produce large amounts of inflammatory molecules that causes neuronal damage in the substantia nigra but not other brain regions, such as in the striatum (Castano, Herrera, Cano, & Machado, 1998; Nadeau & Rivest, 2002). On the other hand, animals deficient in TNFα have exaggerated neuronal death after injury (Turrin & Rivest, 2006). The loss of TNFα leads to decreased microglia activation that later results in over activation of microglia (Turrin & Rivest, 2006). This is similar to the results presented in this thesis where minocycline inhibition of microglia resulted in greater RGC death later in the injury.

9.6 Molecular Changes in Neurons after Neuroinflammation

Microglial activation and neuroinflammation can cause genetic changes and protein expression changes in neurons. BM88 is a neuronal differentiation and

cell cycle exit protein and it was down regulated after optic nerve crush injury. It is possible that the neuronal death that occurs after ONC may occur through cell cycle dysregulation, mitochondrial dysfunction, and/or impaired calcium regulation. BM88 expression changes may be able to predict the functioning and health of the neuron because it was first up-regulated at 4 days after ONC and 2 days after optic nerve transection, which is a more severe injury. There was even more drastic loss of RGCs after injection of HAPI cells into the vitreous or tail vein after ONC than ONC alone. Therefore, it was my hypothesis that there may be down-regulation of BM88 earlier after HAPI cell injection and ONC.

The number of RGCs immunoreactive for BM88 after HAPI cell injection and ONC was lower than the number of RGCs surviving. In addition, after HAPI cell injection and ONC there was no increase in BM88 expression at 4 days after injury, but instead the staining intensity was lower. Interestingly, BM88 was down regulated preceding the loss of RGCs at 14 days after HAPI cell injection without ONC. The number of BM88 expressing cells went back up to the number of Brn3a expressing RGCs that were present 14 days after injection, suggesting that the down-regulation of BM88 without ONC may be temporary if retinas were collected after a longer time point. Therefore, the number of RGCs that express BM88 and the staining intensity of BM88 in RGCs that still expressed it was down-regulated in a pro-inflammatory environment and BM88 expression was sensitive to the severity of the injury. This finding suggests that BM88 is

neuroprotective and its continued expression by RGCs may be a good predictor of neuronal health and functioning.

Other neuronal differentiation proteins, such as ephrin receptor B1 (EphB1), are down-regulated during neuroinflammation (Bonow, Aïd, Zhang, Becker, & Bosetti, 2008). Cell cycle dysfunction is also thought to make neurons more prone to apoptosis and has been demonstrated in animal models of Alzheimer's disease and ischemia (Bonda et al., 2010; Politis, Thomaidou, & Matsas, 2008; Rashidian et al., 2005). Neuroinflammation may play a role in this cell cycle dysregulation because NSAIDs have shown to prevent neurons from re-entering the cell cycle in Alzheimer's mice (Varvel et al., 2009). In addition, the "two hit" hypothesis suggests that oxidative stress and cell cycle dysregulation combined cause apoptosis in neurons (X. Zhu, Lee, Perry, & Smith, 2007; X. Zhu, Raina, Perry, & Smith, 2004). BM88 is also involved in calcium buffering and maintaining the integrity of the mitochondria (Masgrau et al., 2009). Maintaining calcium levels in the neuron is important because calcium overload in the cytoplasm and mitochondria leads to cell death (Orrenius, Zhivotovsky, & Nicotera, 2003). BM88 also inhibits BAX from binding to the mitochondria and preventing apoptosis (Masgrau et al., 2009). Therefore, BM88 is neuroprotective and its down-regulation leads to RGC death.

9.7 Implications of this Research in Glaucoma

Glaucoma is similar to other neurodegenerative disorders since it shares many processes that lead to death of neurons and RGCs (Bayer, Keller, Ferrari, &

Maag, 2002; Gupta & Yucel, 2007; McKinnon, 2003). Many components of glaucoma are seen in other neurodegenerative disorders such as Alzheimer's disease (Hinton, Sadun, Blanks, & Miller, 1986; Pache & Flammer, 2006; Tamura et al., 2006). Neuroinflammation has been shown to be a crucial component of many neurodegenerative disorders and glaucoma. Microglia in certain activation states, at certain times and severity of injury, and depending on whether there is access to the cell bodies in the retina or the axons in the optic nerve result in modulation and death of RGCs. This thesis demonstrated that microglia can be neuroprotective but this is dependent of the time course and location of the injury. This finding needs to be taken into consideration when developing a therapeutic intervention that modulates microglia. Microglia also have important interactions with other cell types and neuroinflammation has other factors that need to be explored further. However, understanding the mechanisms of microglial activation is useful in studying the overall inflammatory response.

9.8 Limitations of the Studies

There are benefits and disadvantages to using immortalized cell culture systems for studying microglia. The benefit of using immortalized cell culture is that it is less time consuming, less challenging technically, and more cost effective than attempting to get pure primary microglial culture. Cultured microglia are able to retain characteristics common to CNS resident microglia, such as phagocytosis, secreting pro-inflammatory cytokines, expression of cell surface antigens, and production of NO (Cheepsunthorn, Radov, Menzies, Reid,

& Connor, 2001; Carol A Colton & Gilbert, 1987; Giulian & Baker, 1986). However, Horvath *et al.* (2008) showed that BV-2, HAPI cells, and primary microglial cell culture have different responses to microglial activation modulators. HAPI and BV-2 microglial cells act similarly to primary microglia when accessing migration, IBA-1 expression, and NO release after modulation with LPS and minocycline (Horvath, Nutile-McMenemy, Alkaitis, & DeLeo, 2008). However, there were differences when TNFα, IL-1β, IL-6, and MCP-1 expression and release were assayed (Horvath et al., 2008). For example, IL-1β (31 KD) is up-regulated in primary microglia after LPS stimulation, however, there was an increase of IL-1β (17 KD) in HAPI cells after LPS stimulation (Horvath et al., 2008). According to Horvath *et al.* (2008) there was not as high of an increase in TNFα, IL-6, and MCP-1 in HAPI cells as in primary microglia. However, another study showed that HAPI cells and primary microglia had similar mRNA expression of TNFα, macrophage inflammatory protein (MIP-1), and chemokine receptors (CXCR3 and CX3CR1) in response to LPS activation (Kremlev, Roberts, & Palmer, 2004). Although it can be argued how closely HAPI cells and primary microglia react to modulators of microglial activation, there may exist differences in the stimulation of these cells. Future studies may want to explore grafting primary microglia instead of HAPI cells to simulate a condition closer to what is seen *in vivo*.

9.9 Future Directions

We have already directly promoted the M1 phenotype in microglia and inhibited the M1 phenotype, but we have not directly promoted the M2 phenotype in microglia. This may be done using zymosan. Zymosan is a polysaccharide from yeast cell walls and has been shown to stimulate the innate immune system (Yin et al., 2003). Zymosan activated macrophages were able to stimulate axon regeneration, however, may lead to neurotoxicity in some conditions (Gensel et al., 2009). Other cytokines that are known to induce the M2 state are IL-4, IL-13, and IL-10 (David & Kroner, 2011). These can be used to activate the HAPI cells into an M2 state and determine if there is neuroprotection after injury.

The loss of RGCs 14 days after HAPI cells were injected into the tail vein or vitreous without injury suggests that phagocytosis and physical proximity is not needed for HAPI cells to cause RGCs to go through apoptosis. To determine if the physical presence of HAPI cells are required for their action on neurons or if there is some secreted factor exerting the effect, we could use a co-culture system. Primary RGC culture can be exposed to media that has been conditioned with treated or untreated HAPI cells. Once we have determined the survival or loss of primary RGCs, the media could be concentrated and fractionated to identify the secreted factors that result in either a neuroprotective or toxic outcome. This concentrated media, with the secreted factors of interest, could be injected into the vitreous or tail vein with ONC to determine if the cocktail is neuroprotective or cytotoxic. This would help provide targets for future

therapeutic substances and help determine when in the injury they are neuroprotective. The novel method of allografting HAPI cells used in this study could be used to test the effect of any substance that modulates the activation of microglia on neuronal survival.

9.10 Figures and Tables

Figure 9.1: RGC survival after injection of untreated, LPS activated, and minocycline treated HAPI cells into the vitreous without ONC. The RGC density was lower 14 days after untreated HAPI cells were injected (blue). There was no change from control (solid grey line) when minocycline treated HAPI cells were injected (green). LPS activation of HAPI cells (red) led to the death of RGCs to levels expected after 7 days of ONC (dotted grey line).

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

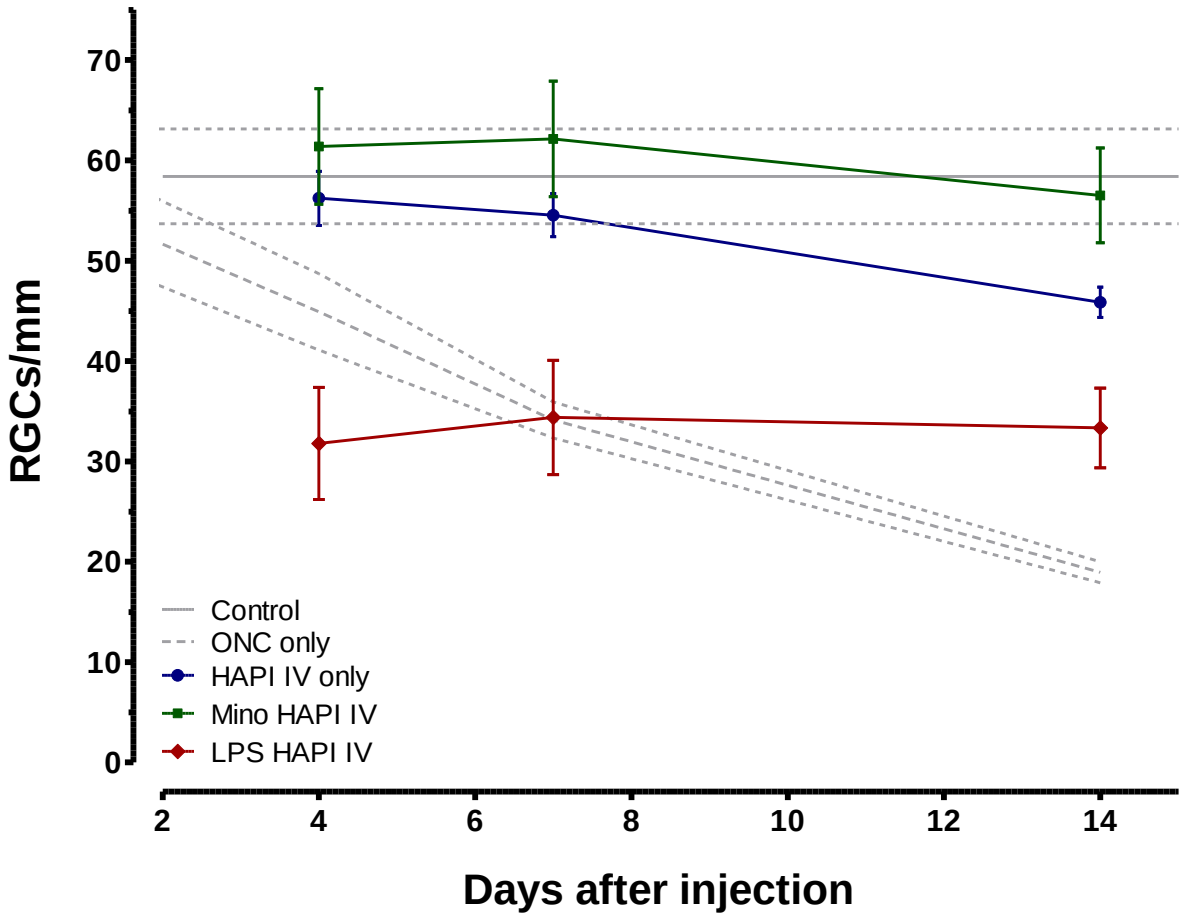

RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after injection
Control
ONC only
HAPI IV only
Mino HAPI IV
LPS HAPI IV

Figure 9.2: RGC survival after injection of untreated, LPS activated, and minocycline treated HAPI cells into the vitreous with ONC. There was a greater loss of RGCs 4 and 7 days after untreated HAPI cells were injected (blue) when compared to the loss expected after ONC alone (dotted grey line). There was no change when minocycline treated HAPI cells were injected (green) compared to the RGC density expected after ONC alone. LPS activation of HAPI cells (red) led to lower RGC survival 4 and 14 days after ONC than what would be expected after ONC alone.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

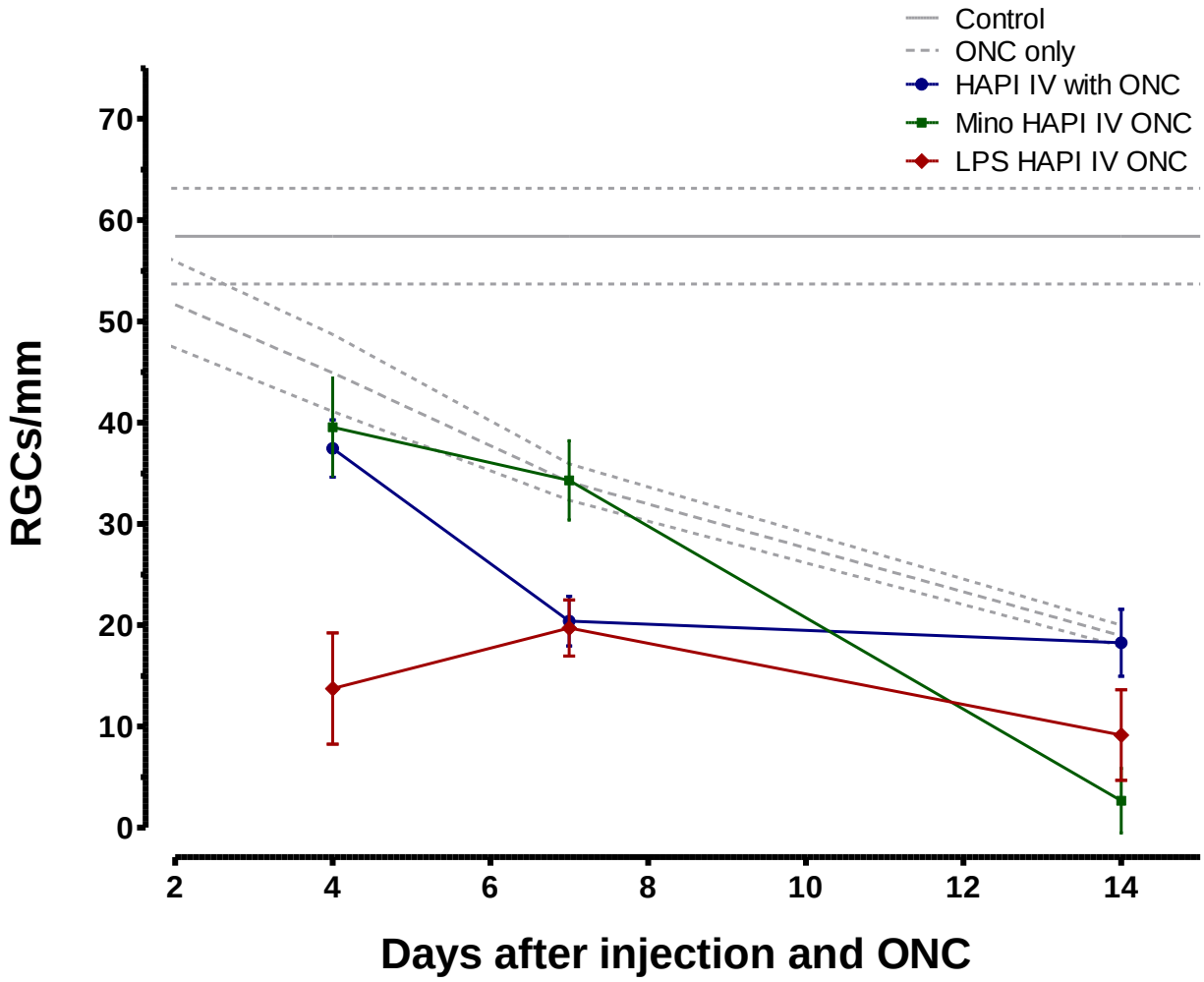

Control
ONC only
HAPI IV with ONC
Mino HAPI IV ONC
LPS HAPI IV ONC
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after injection and ONC

Figure 9.3: RGC survival after injection of untreated, LPS activated, and minocycline treated HAPI cells into the tail vein without ONC. There was a loss of RGCs 14 days after untreated HAPI cells were injected (blue). This loss was not seen after LPS activated (red) or minocycline (green) treated HAPI cells were injected.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.

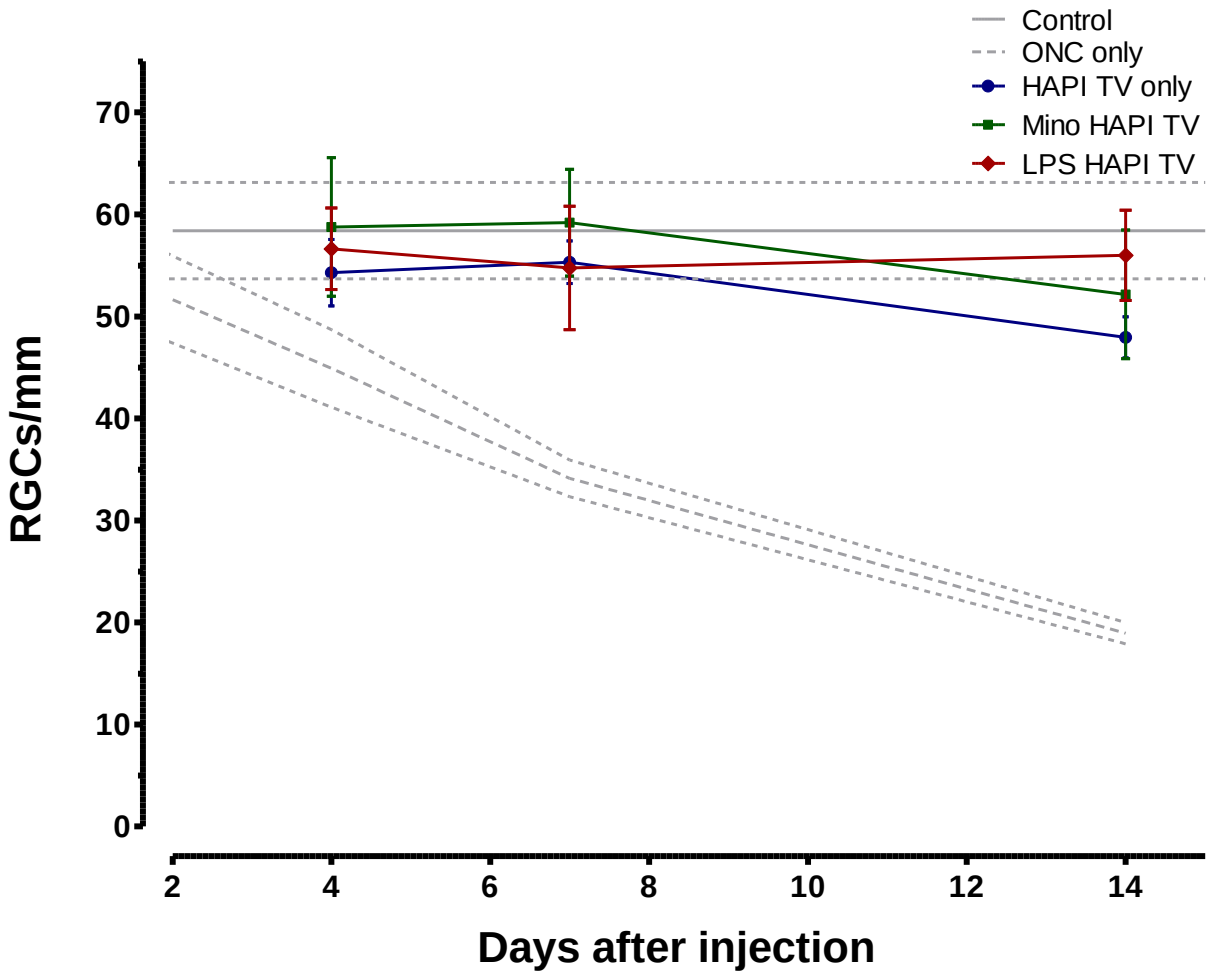
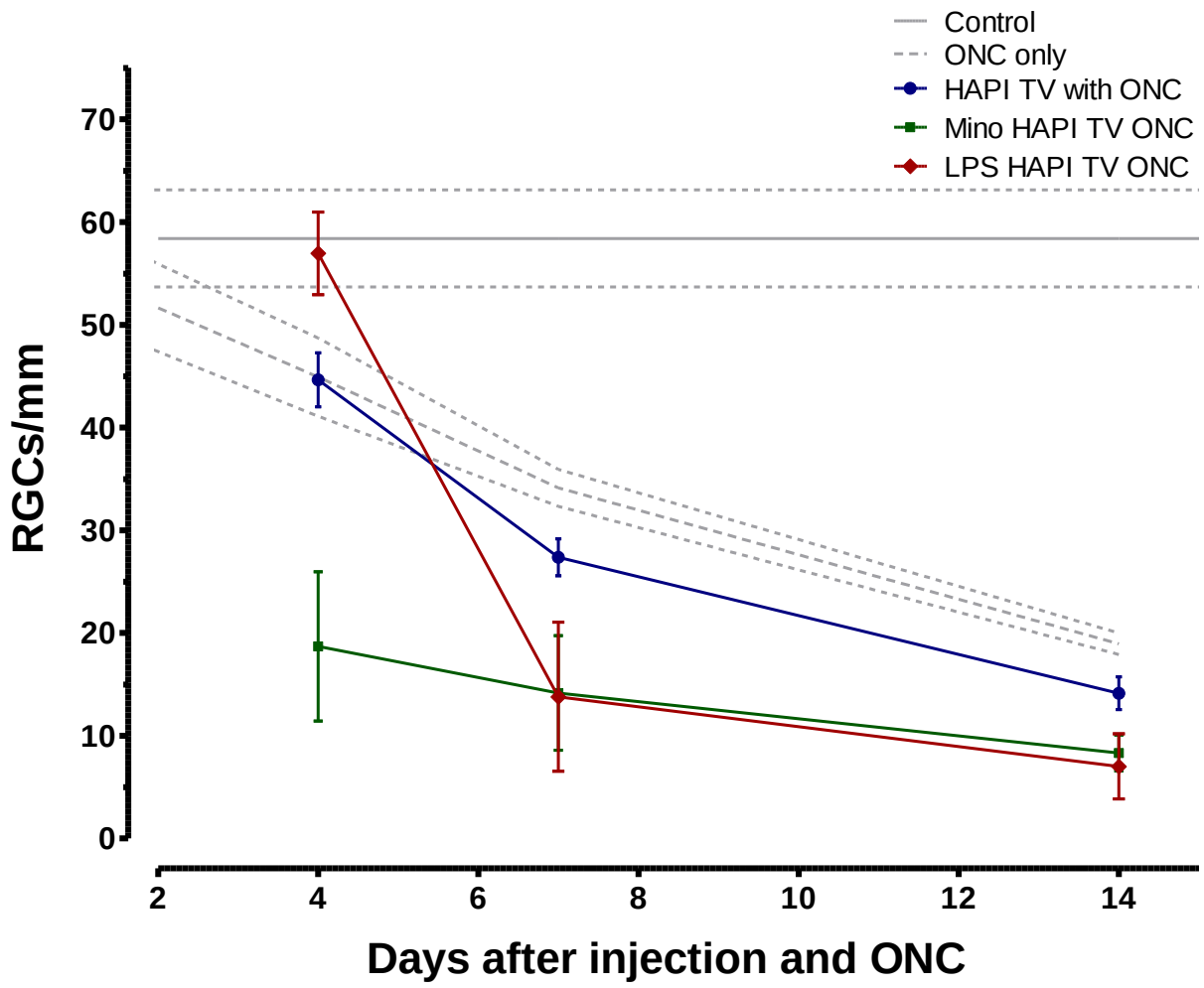

Control
ONC only
HAPI TV only
Mino HAPI TV
LPS HAPI TV
RGCs/mm
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Days after injection

Figure 9.4: RGC survival after injection of untreated, LPS activated, and minocycline treated HAPI cells into the tail vein with ONC. There was a greater loss of RGCs 7 and 14 days after untreated HAPI cells (blue) were injected when compared with the RGC density after ONC alone (dotted grey line). When minocycline treated HAPI cells (green) were injected there was greater RGC loss than would be expected from ONC alone. Early after injury, LPS activated HAPI cells (red) were neuroprotective since there was no loss of RGCs and were statistically the same as controls (solid grey line). However, 7 days after injury, this neuroprotection was lost resulting in greater RGC loss than what would be expected after ONC alone.

Error bars (or dotted lines) = 95% confidence interval. ANOVA statistically analysis and Tukey's post-hoc test were performed.


Control
ONC only
HAPI TV with ONC
Mino HAPI TV ONC
LPS HAPI TV ONC
RGCs/mm
0
10
20
30
40
50
60
70
2
4
6
8
10
12
14
Days after injection and ONC

APPENDIX

Appendix I: Abstract for Chapter 4

Purpose: Microglia are the innate immune cells of the CNS that respond to injury by proliferating and migrating to the affected areas. Activated microglia release nitric oxide, glutamate, and superoxide radicals, which are harmful to retinal ganglion cells (RGCs). They may also benefit surviving cells by removing toxic cellular debris or and secreting neurotrophic factors. The paradoxical role of microglia remains controversial because the nature and time-course of the injury that determines whether microglia acquire a neuroprotective or pro-inflammatory phenotype is unknown. The purpose of this study was to determine if cultured HAPI cells injected into the eye, or systemically, would migrate to the retina and optic nerve after optic nerve injury and how this would affect the survival of RGCs.

Methods: HAPI cells were labelled with Wheat Germ Agglutinin to differentiate them from endogenous microglial cells. Then HAPI cells were injected into the vitreous (30,000 cells) or tail vein (5 million cells). Retinas were examined at 4-14 days following optic nerve crush and the number of surviving RGCs was determined by counting fluorogold labelled cells.

Results: When HAPI cells were injected into the vitreous or tail vein without ONC, there was a decrease in RGC density after 14 days. When the cells were injected into the vitreous with ONC, there was an increased loss of RGCs over the 14 day period until it leveled off at day 14 to expected values after ONC (4 days: 17%; 7 days: 40%). When HAPI cells were injected into the tail vein with an ONC there was increased death of RGCs after 4 days of injury (7 days: 20%; 14 days: 25%).

Conclusion: HAPI cells when injected into the vitreous or tail vein are able to migrate to the retina or optic nerve (respectively) after optic nerve injury. This suggests that optic nerve signals can attract extrinsic microglia to the retina that results in a pro-inflammatory response. By using this novel technique of allografting HAPI cells, we can use different substances to activate the HAPI cells without causing toxicity to the animal and activation of other cells.

Appendix II: Abstract for Chapter 5

Purpose: BM88 (C38; Cend1) is a cell cycle exit and neuronal differentiation protein that is expressed in the rat eye by post-mitotic retinal ganglion cells (RGCs). BM88 labelling has been used as a marker of surviving RGCs after optic nerve injury. Thy1.1 has also been used as a marker for RGC loss after optic nerve transection (ONT) but after optic nerve crush (ONC) the decrease in thy1 expression precedes the loss of RGCs. Differences in the expression of RGC markers may depend on the type of injury. The purpose of this study was to determine if BM88 expression was correlated with RGC loss after ONC and ONT injuries.

Methods: Sprague Dawley rats were injected with fluorogold (FG) in the superior colliculus to label RGCs and received ONC or ONT 3 days later. The eyes were collected at 2-28 days after injury. The eyes were fixed and cryoprotected with overnight. Frozen sections were labelled with BM88 overnight. The RGCs were counted using an epifluorescence microscope. Intensity of the BM88 cell labelling was measured from images standardized for exposure using NIH ImageJ.

Results: In control retinas, 98.9% of BM88 immunoreactive (-IR) RGCs were co-localized with FG. However, there was a significant down regulation of BM88 by RGCs 7 days after ONT (47.7% labelled) and 7 days after ONC (19.6% labelled). By 28 days of optic nerve injury only 10 - 27% of RGCs were labelled with BM88. Not only were there fewer RGCs labeled with BM88 but the staining intensity of the remaining labeled cells was reduced to 41 - 51% of the control after 28 days of optic nerve injury. However, early in the injury (2 days for ONT and 4 days for ONC) there was a significant increase in the staining intensity of BM88.

Conclusion: Nearly all BM88-IR RGCs co-localized with FG labeled RGCs in control retinas. However, both the number of BM88-IR RGCs and their intensity decreased gradually between 4 and 28 days, preceding the loss of FG labeled cells. These findings indicate that BM88 is not a good marker of surviving RGCs but may indicate abnormal RGC functioning.

Appendix III: Abstract for Chapter 6

Purpose: Microglia are the innate immune cells of the CNS and become activated in response to injury or neurodegeneration. They are able to exert neuroprotective and pro-inflammatory effects that may determine the survival of neurons. Microglial activation and neuroinflammation may also change gene and protein expression in neuronal cells. BM88 expression has been shown to be down-regulated after optic nerve crush and may be a good indicator of neuronal dysfunction. It is the purpose of this study was to test if a pro-inflammatory condition in the retina can change the expression of BM88 in RGCs.

Methods: HAPI cells were injected into the vitreous or tail vein with an optic nerve crush. Retinas were examined 4-14 days following the injury and there number of surviving RGCs was accessed by Brn3b labelling. The number of RGCs with BM88 and the intensity of the staining were determined by measurements taken from images standardized for exposure after immunolabelling using ImageJ software.

Results: When HAPI cells are injected into the vitreous without ONC there are 28% less RGCs that express BM88 at 7 days. This precedes the loss of RGCs seen at 14 days after injection. There is also a decrease in staining intensity of BM88 to 53% of the control after HAPI cells are injected into the vitreous. A similar trend is seen after HAPI cells are injected into the tail vein. There were 20.5% less RGCs that express BM88 at 4 days. This preceded the death of RGCs at 14 days after injection. There is a decline in staining intensity of BM88 54% of the control. There are less RGCs that express BM88 than Brn3a after HAPI cells are injected into the vitreous with ONC (4 days: 34% less, 7 days: 58% less, and 14 days: 84% less). This was accompanied by a decline in staining intensity of BM88 by 35% of the control. This was similar after tail vein injection and ONC where there were less BM88 immunoreactive RGCs than Brn3a immunoreactive RGCs (4 days: 43% less and 7 days: 53% less). The staining intensity was 42% of the control.

Conclusion: The expression of BM88 and its staining intensity is down regulated by RGCs in a pro-inflammatory environment and may be sensitive to the severity of the injury. This suggests that BM88 may be neuroprotective and its expression may be a good marker for neuronal health and functioning.

Appendix IV: Abstract for Chapter 7

Purpose: In response to injury, microglia quickly become activated and may acquire a neuroprotective or neurotoxic phenotype depending on the time course and nature of the injury. Lipopolysaccharide (LPS) is a toxin that is isolated from gram negative bacteria. It is thought to put microglia into a cytotoxic state, however, some studies show conditions where LPS activation can be neuroprotective. The problem with using LPS to study the effect of microglial activation on neuronal survival *in vivo* is that LPS also activates other cell types. The purpose of this study was to activate HAPI microglial cells *in vitro* and evaluate the effect on retinal ganglion cell (RGC) survival after optic nerve injury when the activated HAPI cells were injected back into the animal.

Methods: HAPI cells were labelled with Wheat Germ Agglutinin to differentiate them from endogenous microglial cells. HAPI cells were activated using 1 µg/mL of LPS for 24 hours in the culture media. Then HAPI cells were injected into the vitreous (30,000 cells) or tail vein (TV) (5 million cells) of female Sprague Dawley rats. Retinas were examined at 4-14 days following optic nerve crush (ONC) and the number of surviving RGCs was determined by counting Brn3b immunoreactive cells.

Results: The RGC density was not significantly different from control retinas when LPS activated HAPI cells were injected into the TV without injury. The RGC density 4 days after injection of LPS activated HAPI cells with ONC was not different from controls. However, 14 days after ONC and injection of LPS activated HAPI cells into the TV, the RGC density was 51.7% lower than the RGC density 14 days after ONC alone. The RGC density after injection of LPS activated HAPI cells into the vitreous was significantly lower than the control (4 days: 42.2%, 7 days: 37.5% , 14 days: 39.4%). This was similar to the RGC death seen 7 days after ONC. The RGC density after injection of LPS activated HAPI cells into the vitreous with ONC was significantly lower than the density after ONC alone (4 days: 69.4%, 7 days: 42.2%, 14 days: 51.7%). The RGC density was also lower than when HAPI cells were injected into the vitreous with ONC at 4 days (63.3%) and 14 days (49.9%).

Conclusion: LPS activated HAPI cells were neuroprotective early after ONC injury when injected into the tail vein, however, they were neurotoxic later in injury. When LPS activated HAPI cells were injected into the vitreous, a pro-inflammatory response resulted, leading to increased RGC death. These findings suggest that the proximity of activated microglia to the injury site and time course of microglial activation determines if they acquire a neuroprotective or cytotoxic phenotype.

Appendix V: Abstract for Chapter 8

Purpose: The roles of activated microglia after injury is disputed due to studies that show that microglia have both neuroprotective and cytotoxic tendencies. What may determine what phenotype microglia take may depend on the time course and nature of the injury. Minocycline is a second generation tetracycline that was originally used for its antimicrobial properties. It is now being investigated for its anti-inflammatory properties and is used experimentally to modulate microglia. The issue becomes that minocycline modulates other cell types, like lymphocytes and RPE, also. It has also become controversial since experimental data has suggested that its use is neuroprotective after neuronal injury and disease, but some clinical trials have shown harmful effects. The purpose of this study is to study the effect of minocycline on microglia and determine how microglia modulated with minocycline affect RGC survival.

Methods: HAPI cells were labelled with wheat germ agglutinin Texas red and activated using 10 µg/mL of minocycline for 1 hour in the culture media. Then HAPI cells were injected into the vitreous (30,000 cells) or tail vein (TV) (5 million cells) of female Sprague Dawley rats. Retinas were examined at 4-14 days following optic nerve crush (ONC) and the number of surviving RGCs was determined by counting Brn3b immunoreactive cells.

Results: When minocycline treated HAPI cells were injected into the tail vein or vitreous without injury to the optic nerve, the RGC density was not different from controls over the 14 day period. The RGC survival after minocycline activated HAPI cells were injected into the tail vein with optic nerve crush was lower than after ONC alone (4 days: 58.4% less, 7 days: 58.5% less, and 14 days: 56.1% less). There was also less RGC survival 4 and 7 days after minocycline treated HAPI cells were injected into the tail vein with ONC (4 days: 58.1% less and 7 days: 48.3% less) but not after 14 days of injection and ONC. After intravitreal injection of minocycline treated HAPI cells with ONC there was no difference in the RGC density when compared to that of the ONC alone at 4 and 7 days after injection. However, 14 days after intravitreal injection there was a loss of 56.1% more RGCs than that expected from ONC alone.

Conclusion: Minocycline has protective and destructive effects depending on the time course of the injury and the location where microglia are being affected.

Appendix VI: Summary of Key Experiments

Condition	Mode of Injection	ONC?	Days after injection	% Loss Greater than Control	Neuroprotective/ Cytotoxic
Untreated HAPI Cells	Intravitreal	No	4	ns	No effect
			7	ns	
			14	19%	Cytotoxic
		Yes	4	17%	Helps clean up dead and dying cells
			7	40%	
			14	ns	
	Tail Vein	No	4	ns	No effect
			7	ns	
			14	12%	Cytotoxic
		Yes	4	ns	Cytotoxic
			7	20%	
			14	25%	
LPS activated HAPI Cells	Intravitreal	No	4	42%	Cytotoxic
			7	38%	
			14	39%	
		Yes	4	69%	Cytotoxic
			7	42%	
			14	52%	
	Tail Vein	No	4	ns	No effect
			7	ns	
			14	ns	
		Yes	4	*18% more cells than control	Neuroprotective
			7	60%	Cytotoxic
			14	52%	
Minocycline treated HAPI Cells	Intravitreal	No	4	ns	No effect
			7	ns	
			14	ns	
		Yes	4	ns	Neuroprotective
			7	ns	
			14	56%	Cytotoxic
	Tail Vein	No	4	ns	No effect
			7	ns	
			14	ns	
		Yes	4	58%	Cytotoxic
			7	59%	
			14	56%	

ns = not significant

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