Inflammation and Neuroplasticity in the Cornea.

By
Sasha Rhoslyn Walsh Power

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Science Honors Biology.

September, 2015, Halifax, Nova Scotia

Approved: Dr. Anna-Maria Szczesniak
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Signature Page

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TABLE OF CONTENTS

LIST OF FIGURES

Development of a Mouse (*Mus musculus*) Model of Post Injury Neuroplasticity in the

Cornea

by Sasha Rhoslyn Walsh Power

Abstract

Neuroplasticity is related to the addition of additional axon collaterals and dendrites between a nerve cell and one it has previously shared a connection in order to strengthen, re-establish or forge brand new connections with pre-existing neurons. The role of neuroplasticity following a wound to an epithelium such as the cornea is of great importance to vision science, as it may help shape future medical approaches to common eye injuries in such a way that chronic eye pain and even blindness can be avoided. The present study suggests that *Mus musculus* is a viable model for the study of changes in nerve morphology and density that are correlated with neuroplasticity. Furthermore, the work presented here suggests a possible correlation between peak immune cell infiltration of the cornea and the initiation of changes in nerve density and organisation related to neuroplasticity. After significant ($P<0.05$) decrease in the density of both major nerves and neuronal processes following chemical cauterization with silver nitrate, the density of both major nerves and nerve processes increased a significant ($P>0.05$) amount when mice were allowed to recover for up to 48 hours after treatment. Interestingly, nerve processes increased by 31.4% in only 48 hours. These results suggest that neuroplasticity is observed in the cornea following an injury. This study also shows that macrophage and neutrophil infiltration of the stroma peaks at around the same time that the cornea begins to regain some nerve density, suggesting that there may be a link between these two wound healing processes in the corneas of mice and potentially other mammals, including humans.

08-09-2015

ACKNOWLEDGEMENTS

I. INTRODUCTION

The cornea is the outermost structure of the eye, working to protect the eye from infection and injury as well as acting as a refractive structure that is vital to the sense of sight (Delmonte and Kim, 2011). It is the most innervated epithelium in the body, being 600 times more innervated than skin (Marfurt and Ellis, 1993). An important feature of the cornea is its transparency, which allows light to pass through it and be focused in the eye such that sight is enabled (Delmonte and Kim, 2011; Yu and Rosenblatt, 2007). This transparency can be attributed to a number of factors including cellular anatomy. Furthermore, the shape of the cornea as a whole is a vital component of the vision system and any physical defects that alter the surface shape or the opacity of the cornea, such as scarring or ulcers, will have detrimental effects on the vision of the specimen (Lambiase *et al.* 1998).

Damage to the cornea can permanently alter the structure of the cornea and the cornea's sensitivity to painful stimuli, negatively impacting the vision and comfort of the individual (Lambiase *et al.* 1998). Immediately following damage to the cornea, the individual is going to experience pain, inflammation and immune cell activity in the cornea (Akpek and Gottsch 2003; Belmonte *et al.*, 2003) As the wound penetrates increasingly deep cellular layers of the cornea these immediate effects worsen, become more complex, and the likelihood of long term damage to the cornea increases (Belmonte *et al.*, 2003). Such long term effects of corneal damage include corneal scarring, vision impairment, constant burning and other dry eye related symptoms and hypersensitivity to painful stimuli (Belmonte *et al.*, 2003; Yu *et al.*, 2008). Furthermore, the hypersensitivity

7

and increased response to painful stimuli, a phenomenon called hyperalgesia, a subdivision of neuropathic pain, is thought to originate from damage to or malfunction of the nerves such as those in the cornea (Moulin *et al.,*2007). It is believed that sensitization develops because injury to the tissue results in the release of endogenous inflammatory molecules from damaged cells and the immune cells that subsequently infiltrate the cornea mediate or possible induce a number of short term and long term changes (Belmonte *et al.*, 2003). It is hypothesized that receptor proteins on the surface of the neurons are directly affected by secretions of immune cells during the inflammatory response, leading to potentially harmful and even permanent changes to the neuron's form and function (Belmonte *et al.*, 2003). Furthermore, it is interesting to note that damage to corneal nerve leashes and the nerve plexus has been shown to significantly impair corneal healing (Yu *et al.,* 2008).

The link between the inflammatory response in the cornea and reorganisation of corneal nerves, and their subsequent connection to hyperalgesia in the cornea is not well understood (Belmonte *et al.*, 2003). It is thought that characterising these mechanisms of interaction and action may one day allow for hyperalgesia in the cornea to overcome, relieving many of prolonged suffering following an injury to the cornea. Obtaining a better understanding of the link between changes in nerve structure during an inflammatory response to an injury with the onset of hyperalgesia in the cornea is the driving force of the work presented here.

1. The Cornea

Structural Overview

The cornea is the outermost structure of the eye, working to protect the eye from infection and injury as well as acting as a refractive structure that is vital to the sense of sight (Delmonte and Kim, 2011). It is the most innervated epithelium in the body, being 600 times more innervated than skin (Marfurt and Ellis, 1993). Interestingly, the cornea contains the same types of nerve cells, called nociceptors (primary sensory nerves that mainly mediate pain), as the skin (Wenk and Honda, 2003; Armentia, Cabanes, and Belmonte, 2000; Belmonte *et al.*, 2003). These corneal nerves exist in a simple avascular tissue (Wenk and Honda, 2003; Armentia, Cabanes, and Belmonte, 2000; Belmonte *et al.*, 2003). Additionally, the free nerve endings in the cornea are mere microns from the surface, making visualization easy and allowing for more direct access to the neurons for things such as rapid and consistent topical treatment of compounds (Wenk and Honda, 2003).

An important feature of the cornea is its transparency, which allows light to pass through it and be focused in the eye such that sight is enabled (Delmonte and Kim, 2011; Yu and Rosenblatt, 2007). This transparency can be attributed to a number of factors including cellular anatomy. The shape of the cornea as a whole is a vital component of the vision system and any physical defects that alter the surface shape or the opacity of the cornea, such as scarring or ulcers, will have detrimental effects on the vision of the specimen (Lambiase *et al.* 1998). Any deviations in the thickness of the cornea, and any lack of uniformity in the cornea's layers can distort the way the individual views the

world around them, causing issues such as white spots in their field of view, near- and farsightedness, and so forth (Lambiase *et al.* 1998).

The cornea as a whole is prolate – meaning it is flatter on the periphery and has a steeper curve in the middle, and has a wider horizontal diameter than vertical diameter (Delmonte and Kim, 2011). This prolate shape allows for vision as we know it (Delmonte and Kim, 2011). The shape is maintained by the rigid stromal layer in the anterior portion the cornea, which is composed of actin bundles (See Figure 1) (Delmonte and Kim, 2011). Unfortunately the difference in rigidity between the anterior and posterior stroma means that the posterior stroma is more sensitive to shearing, folding, cuts and damage in general, making the posterior portion of the cornea a likely site for injury. (Delmonte and Kim, 2011; Wenk and Honda, 2003).

The cornea comprises of three cellular layers and two interfacial membranes in addition to corneal nerves, which will be discussed later (Delmonte and Kim, 2011) (See Figure 1 for a visual of corneal structure). The outermost layer is the epithelium; a stratified, non keratinous, squamous layer that is uniform all the way around (Delmonte and Kim, 2011). The epithelium works with the tear film covering the eye to protect the eye from chemical and mechanical damage as well as providing over two thirds of the refractive power of the eye (Delmonte and Kim, 2011). The deepest layer of cells in the epithelium is the basal layer, which contains the only corneal epithelial cells capable of mitosis (Delmonte and Kim, 2011). As these cells divide previous layers are pushed up and flattened in a fashion similar to human skin cells (Delmonte and Kim, 2011). Beneath the epithelium there is an interfacial membrane called the Bowman layer that is non-

cellular and helps maintain cornea shape, but is incapable of healing damage and scars if injured (See Figure 1) (Delmonte and Kim, 2011).

Posterior to the Bowman layer is the stroma, a collagen containing structure responsible for much of the shape, strength and transparency of the cornea (Delmonte and Kim, 2011). The stroma is comprised of layers of collagen fibers bound in little bundles called fibrils that all run parallel to each other, while each layer runs perpendicular to the layer above and below it, scattering light in such a way that the cornea appears transparent and is still exceptionally strong (Delmonte and Kim, 2011). Keratocytes are also present in the stroma, making collagen and other molecules that are important for stromal homeostasis (Delmonte and Kim, 2011). The Descemet's membrane is posterior to the stroma, and is produced by the endothelium (Delmonte and Kim, 2011). It is amorphous in shape and considered vital to corneal function. The deepest layer of the cornea is the endothelium, a single layer of cells of uniform shape that arrange in a honeycomb like pattern and function to maintain a specific level of turgidity in the cornea in order to maintain vision clarity (Delmonte and Kim, 2011). These cells have no mitotic ability and their number decreases with age, disease, inflammation and physical trauma (Delmonte and Kim, 2011). Additionally it should be noted that the cornea is avascular; it relies on the diffusion of blood components and oxygen, which are supplied by tiny vessels at the edge of the cornea and blood components brought in the tear film (Delmonte and Kim, 2011).
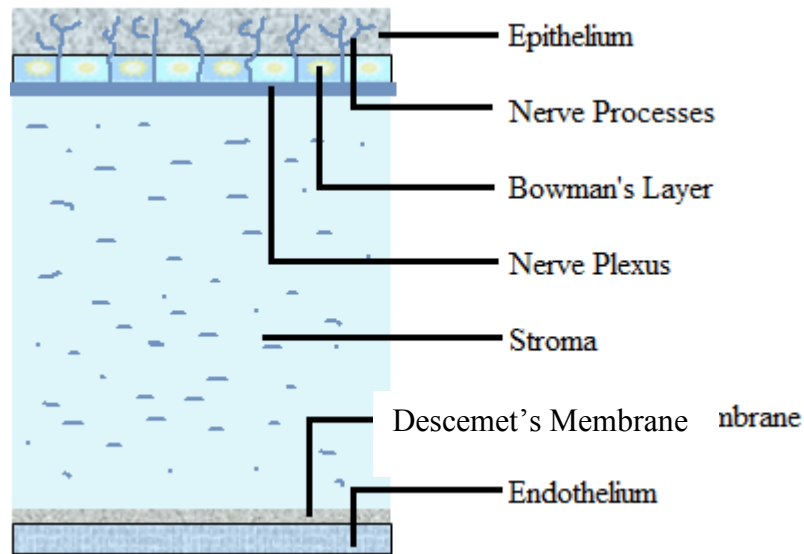
Figure 1. Organisation of Cellular Layers in the Typical Mammalian Cornea. Altered

from Delmonte and Kim, 2011.

Corneal Nerve Structure

The cornea is the most innervated epithelium in the body (Marfurt and Ellis,

1993). Despite this, innervation of the cornea lacks complexity (Wenk and Honda, 2003).

The nerves of the cornea originate from a few primary sensory neurons found in the

ipsilateral trigeminal ganglion (de Felipe *et al.*, 1999). These neurons are primary sensory

neurons whose peripheral axons end as free nerve endings in the outermost layers of the

corneal epithelium (Armentia *et al.*, 2000). They appear to be nociceptive, because

irritation and pain are the only sensations that are known to be evoked by a variety of

stimuli (Armentia *et al.*, 2000). Axons of neurons at the periphery of the cornea lose their

myelin sheath upon entering the stroma (Müller *et al.,* 2003). In the stroma the neurons are grouped in nerve bundles that are radially oriented and do not have a set pattern of numbers in each bundle (Belmonte *et al.*, 2004). These bundles branch extensively, forming a nerve plexus beneath the epithelium that then turn ninety degrees as the nerve extensions reach up into the epithelium after crossing Bowman's layer (See Figure 2) (Belmonte *et al.*, 2004). Within the epithelium the neuron branches are parallel to the corneal surface (Müller *et al.,* 2003).

The axons of corneal nerves look the same when viewed with classical microscopy techniques and electron microscopy, but appear to be functionally different types (Belmonte *et al.,* 2003). The corneal neurons are differentiated structurally; they are called A-delta type if they have thin layers of myelin, or C type if they have no myelin around the axon (Belmonte *et al.*, 1997; de Felipe *et al.,* 1999). These nerves differ in function by the intensity of sensation needed to activate each nerve type, as well as their response profile (Belmonte *et al.*, 1997; de Felipe *et al.,* 1999). In the mouse, the A-delta type nociceptor neurons account for 30% of corneal neurons while the rest are C type neurons (Belmonte e*t al.,* 1997; de Felipe *et al.,* 1999).

Both A-delta and C type neurons respond to the presence of a stimulus, but do not encode duration, and barely provide any information on the intensity (Belmonte *et al.,* 2004). Interestingly, they are more sensitive than the same receptor types in skin (Armentia *et al.*, 2000; Belmonte *et al.*, 2003). Furthermore, A-delta and C type neurons act as nociceptors in the cornea, and can thus be further subdivided by the type of stimuli that the neurons respond to (Armentia *et al.*,2000; Belmonte *et al.*, 2003).

13

Electrophysiological studies have shown that the acute and sharp pain associated with mechanical pressure on the cornea are likely due to mechano-nociceptors, while the polymodal nociceptors are more responsive to heat and chemical irritation (Armentia *et al.*,2000; Belmonte *et al.*, 2003). Lastly, cold-sensitive neurons respond predominantly to cooling of the corneal surface (Armentia *et al.*,2000; Belmonte *et al.*, 2003).

Injury of the Cornea

Immediately following damage to the cornea, the individual is going to experience pain, inflammation and immune cell activity in the cornea (Akpek and Gottsch 2003; Belmonte *et al.*, 2003) As the wound penetrates increasingly deep cellular layers of the cornea these immediate effects worsen, become more complex, and the likelihood of long term damage to the cornea increases (Belmonte *et al.*, 2003). Such long term effects of corneal damage include corneal scarring, vision impairment, constant burning and other dry eye related symptoms and hypersensitivity to painful stimuli (Belmonte *et al.*, 2003; Yu *et al.*, 2008). Damage to corneal nerve leashes and the nerve plexus has been shown to significantly impair corneal healing (Yu *et al.,* 2008). Furthermore, the hypersensitivity and increased response to painful stimuli, a phenomenon called hyperalgesia, a subdivision of neuropathic pain, is thought to originate from damage to or malfunction of the nerves in the cornea (Moulin *et al.,* 2007). Sensitization is a decrease in impulse firing threshold, enhanced responsiveness, and spontaneous activity of the neuron (Belmonte *et al.*, 2003). It is believed that sensitization develops as a result of release of endogenous

inflammatory molecules from damaged cells, and the immune cells that subsequently infiltrate the cornea (Belmonte *et al.*, 2003). Receptor proteins on the surface of the neurons are thought to be directly affected by secretions from the immune cells during the inflammatory response, leading to potentially harmful and even permanent changes to the neuron's form and function (Belmonte *et al.*, 2003). These interactions and hyperalgesia in general are not well understood in the cornea (Belmonte *et al.*, 2003). It is thought that characterising the mechanisms underlying development of hyperalgesia in the cornea may allow for development of treatment(s), relieving many of prolonged suffering following an injury to the cornea.

Causes of insult and injury to the cornea outside of a laboratory setting include disease, accidental exposure to excessive mechanical pressure, shearing force, bacterial infections, noxious amounts of heat or cold and chemical irritants (Stepp et al., 2014). Interestingly, the increasingly popular vision corrective surgery called LASIK (laser in situ keratomileusis) has been reported to damage corneal nerves such that severe dry eye symptoms are induced, including constant burning and itching of the eyes that persist for over six months in 18-41% of all operations (Chao *et al.,* 2014). Work by Chao *et al.* suggests that understanding the role of corneal nerve healing after damage by LASIK may save a lot of people from such torment (Chao *et al.,* 2014). It is important to note that in all of these circumstances the immune system is involved in the wound healing process (Chao *et al.,* 2014; Strepp *et al.* 2014; Lambiase *et al.*, 2000; Wenk and Honda, 2003) This involvement will be considered in this study as well.

An interesting instance of a discovery in the field of corneal neuroplasticity involves the work done by Lambiase *et al.* in 1998 and 2000. This group was studying neurotrophic ulcers in the cornea caused by diseases such as fifth-nerve palsy, chemical burns, viral infections, surgery of the cornea, abuse of topical anesthetics, diabetes mellitus, and multiple sclerosis, among others (Lambiase *et al.*, 1998). These ulcers result from death of sensory nerves in the cornea, and can result in progressively worse corneal damage, opacification and even perforation and vascularisation of the cornea (Lambiase *et al.*, 1998).

The Cornea and the Immune System

The cornea is vital to vision, as its transparency provides most of the eye's refractive power (Akpek and Gottsch, 2003). Furthermore, the cornea functions as a barrier that keeps pathogens and other sources of damage from reaching the internal structure of the eye and the optic nerve (Akpek and Gottsch, 2003). The ability of the cornea to recognize sources of damage, such as pathogens or noxious chemicals, as foreign matter and eliminate them is vital in maintaining the transparency of the cornea and therefore critical to preserving vision (Akpek and Gottsch, 2003). The cornea is constantly exposed to the exterior world and lacks vasculature (Akpeck and Gottsch, 2003). Interestingly, the cornea is "immune separated" from the rest of the body, meaning most immune and

16

protective cells and substances come from nearby tissues like the conjunctiva ofthe eye,

often resulting in a time delay between exposure to a damaging substance and the

mounting of an immune response (Akpek and Gottsch, 2003). Nevertheless, the cornea

exhibits both innate and acquired defenses against damage (Akpek and Gottsch, 2003).

Innate immunity is the first line of defense against corneal damage or infection and is

present at birth (Alberts *et al.,* 2002). Innate immunity acts as a nonspecific surveillance

system and is composed of physical, molecular and cellular elements (Akpek and

Gottsch, 2003). Physical barriers such as the orbit and eyelid are external to the cornea

and guard against trauma, often in the form of some mechanical insult that may

compromise the surface of the cornea. The molecular components of the cornea's innate

defense system include the tears, the series of effector and regulatory proteins known as

the complement, and a group of proteins known as interferons (Akpek and Gottsch,

2003). Tears flush particulate matter from the corneal surface, prevent drying of the

cornea and transport antimicrobial proteins such as lysozyme and immunoglobin in order

to neutralize some viruses and prevent bacteria from binding to the corneal epithelium

(Akpek and Gottsch, 2003). The complement resides mostly in the periphery of the

cornea and acts to regulate numerous immune activities (Akpek and Gottsch, 2003).

Interferons are made by invading immune cells such as leukocytes and fibroblasts and are

known to induce generalized antiviral state and major histocompatibility complex (MHC)

class one molecules in order to recruit natural killer (NK) cells (Akpek and Gottsch,

2003).

The cellular components of the innate immune system are the epithelial cells of the cornea, corneal nerves, neutrophils, eosinophils, macrophages and Natural Killer cells (Akpek and Gottsch, 2003). Epithelial cells are known to actively participate in the immune response by releasing cytokines that activate immune defenses when a foreign bodyis detected (Akpek and Gottsch, 2003). Corneal epithelial cells also play a passive role in protecting the eye by storing a compound called interleukin (IL)-1α that leaks out when the epithelial cells rupture and leads to immune invasion. In addition IL-1α has also been shown to stimulate growth of blood vessels within the cornea; this leads to the destruction of the cornea and vision (Niederkorn *et al.*, 1989). Corneal nerves are involved in the innate immunity of the cornea because they relay sensory information that leads to reflexive movements that protect the eyes, such as blinking and closing the eyes (Akpek and Gottsch, 2003). Furthermore, the sensation of pain is believed to be related to the release of two neuropeptides, called calcitonin gene related protein and substance P, by the axon terminals of the sensory neurons of the cornea, resulting in the production of cytokines by the corneal epithelial cells and future influx of neutrophils (Akpek and Gottsch, 2003).

Neutrophils, an important player in corneal immune response, exist in the cornea in small numbers under normal physiological conditions. However after corneal epithelial cells are damaged they signal for the neutrophils to invade the epithelium by diapedesis, or adhesion to vascular endothelial cells (Akpek and Gottsch, 2003). The neutrophils proceed to phagocytise a variety of foreign matter (Akpek and Gottsch, 2003). This aspect of the immune response is of particular importance to the work presented here, as

it is believed that neutrophils may directly interact with nerves damaged during a wounding event (Chao *et al.,* 2014; Strepp *et al.* 2014; Lambiase *et al.*, 2000) Also, prolonged permeability of nearby vasculature to neutrophils can lead to blood accumulating at the site of injury, inhibiting vision (Kaiser, 2012) Furthermore, lysosomal contents from neutrophils that are discharged can result in the destruction of healthy tissue, resulting in scar tissue formation that is detrimental to the specimen's vision (Kaiser, 2012)

Another important immune cell in the cornea, the macrophage, has the ability to phagocytise foreign matter, secrete inflammatory cytokines, and possess antigen presenting abilities that are important for the involvement of NT cells (Akpek and Gottsch, 2003). It was previously believed that macrophages were not found in the cornea and instead invade via the tears, however there is recent evidence that macrophages are present naturally in the corneas of rats (Wenk and Honda, 2003).

The innate immune response is quick to respond to an insult to the cornea, however if the damage is too extensive, around twenty-four hours after injury the acquired immune response is initiated (Akpek and Gottsch, 2003). The acquired response is characterized by a pathogen specific response, as compared to non-specific innate immunity (Akpek and Gottsch, 2003). The most notable difference between the acquired and innate defenses is the involvement of Langerhans cells, an essential sentinel cell that typically resides in the periphery of the cornea and plays a vital role to corneal immune surveillance by recognising, processing and presenting antigens (Akpek and Gottsch,

2003). The Langerhans cells then recruit T cells and more macrophages for pathogen

destruction, depending on the specific pathogen at hand (Akpek and Gottsch, 2003).

Unfortunately, the immune response can sometimes be out of proportion to the threat,

leading to irreversible scarring and disorganization of the extracellular matrix of the

cornea that can result in permanent vision loss (Akpek and Gottsch, 2003).

2. Neuroplasticity

Neuroplasticity Versus Neuroregeneration

The ability to understand, characterize and eventually alter the degree to which a

neuron is remodeled in a mature nervous system is of immense importance to number of

fields ranging from modern medicine to the study of learning and memory formation or

loss (Harris and Purves, 1989). The study of mature nervous system reorganisation is a

broad topic that encompasses contributions from the fields of molecular biology, anatomy

and physiology, genetics, neuroscience and cellular biology. A more refined

understanding of the body's response to neural injury and the ability to change neural

"maps" may one day allow humans to surpass the limitations of our current bodies in

ways only written about in science fiction works (Harris and Purves, 1989). Nerve

regrowth and reorganisation after injury are observed in tissues such as the cornea (Yu *et*

al. , 2007; Yu *et al.*,2009; Lambiase, 2000),spinal cord (McKinley *et al*., 1987), the motor cortex in monkeys (Jenkins and Merzenic, 1987), and in most peripheral tissues (Lambiase, 2000).

Neuroregeneration and neuroplasticity are related but not synonymous concepts (Enciu *et al.* 2011). Neuroregeneration is more complex, as it describes the genesis of new neurons from precursor or stem cells that were added or already present in the organism that then proceed to differentiate, integrate into previously existing neural networks and subsequently survive (Johansson, 2007). This concept has three major components: neuron restoration and plasticity, neurogenesis and endogenous protection (Johansson, 2007, Enciu *et al.,* 2011). These components are linked by the action of neurotrophins, growth factors, and stem or precursor cells (Johansson, 2007; Enciu *et al.,* 2011).

Neuroplasticity is related to the addition of axon collaterals and dendrites between a nerve cells which it has previously shared a connection (See Figure 2 for a visualisation of an axon and dendrite relative to the rest of a neuron and the cells often associated with neurons) (Cajal, 1894; Enciu *et al.*, 2011). This is done in order to strengthen or re-establish previous connections, or to forge new connections between pre-existing cells (Cajal, 1894; Enciu *et al.*, 2011). Neuroplasticity, also called "neural plasticity", is where the bulk of research has been done, as it is present in tissues that are easy to study relative to the brain and spinal cord, and because such nerve remodelling is vital to wound healing (Campbell, 2008; Chen *et al,.* 2002). The first description of neuroplasticity appears in a French paper written in 1894 which, upon translation, describes neural plasticity as

21

"associations already established among certain groups of cells would be notably reinforced by means of the multiplication of the small terminal branches of the dendritic appendages and axonal collaterals; but, in addition, completely new intercellular connections could be established thanks to the new formation of [axonal] collaterals and dendrites" (Cajal, 1894). This definition still holds today, although there is some mild disagreement about the use of these terms to describe similar phenomena in the central versus peripheral nervous system (Enciu *et al.*, 2011; Campbell, 2008).
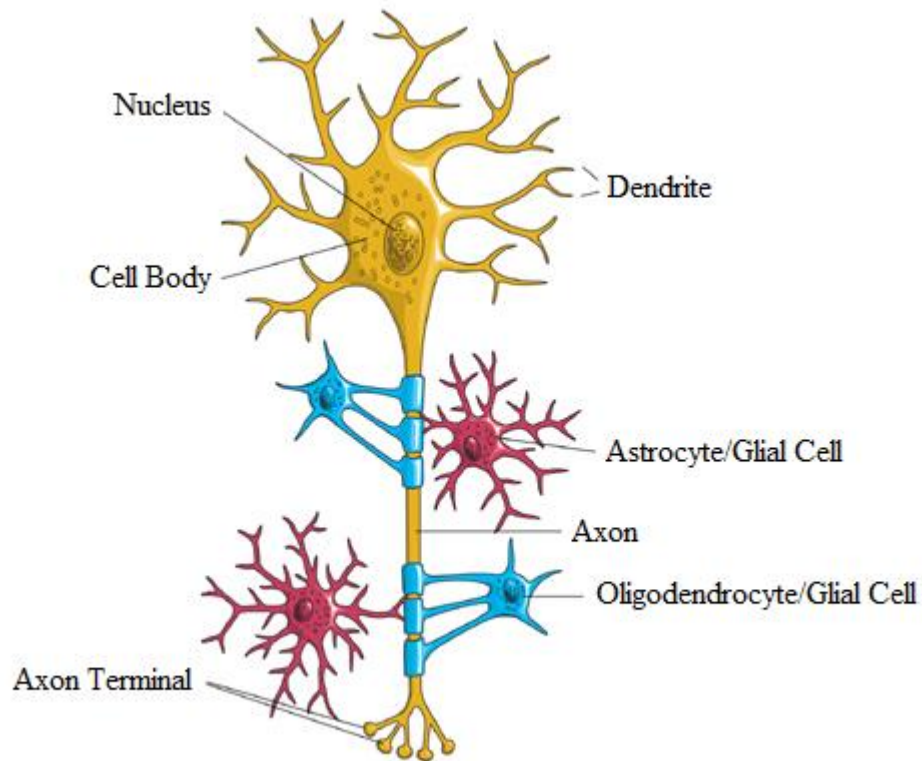
Figure 2. Neuron anatomy, presented with the glial cells often associated with neurons. Sensory information in the form of a stimuli or a neurotransmitter is received by the neuron in the dendrites and then travels down to the axon as an action potential before being passed to the dendrites of the next neuron. This unidirectional mode of information transmission from dendrite to axon is important to understanding the potential impacts severing an axon will have on sensation of the affected tissue. Adapted from National Institute of Neurological Disorders and Stroke (NINDS) (2014).

Neuroplasticity in the Central and Peripheral Nervous System

There is a significant amount of interest in the ability to harness the powers of neuroplasticity in the brain and spinal cord in order to one day overcome the limitations of the human central nervous system as we know it or to rid the world of nerve destroying diseases such as Alzheimer's Disease. When there is an injury to nerves in the brain or spinal cord (the central nervous system) the injured nerve dies off, and it is neuroplasticity of surrounding intact neurons that allow them to branch in new ways to perform the function of the damaged area (Burnett and Zager, 2004). This loss of function of the original area means that the central nervous system does not repair itself (Burnett and Zager, 2004). On the other hand, if a neuron that is innervating the organs, limbs or sensory tissues (and is therefore part of the peripheral nervous system) is damaged, it repairs itself by healing the damaged axon stump and growing new dendrites, often in new areas, thus engaging in neural plasticity differently than in the central nervous system (Burnett and Zager, 2004).

If a neuron that is a part of the peripheral nervous system is injured in some way such that the axon is either partially or completely cut, torn or crushed, the growth of new extensions start almost immediately (Campbell, 2008; Navarro *et al.,* 2007). In humans, this regeneration stabilizes in rate after three to four days at about 2-3 millimeters^2 of new growth a day (Navarro *et al.,* 2007), and has secondary effects such as the induction of plastic changes in the wiring of related areas of the brain cortex and spinal cord (Campbell, 2008; Navarro *et al.,* 2007). The aforementioned processes include the release of neurotrophic factors like those discussed previously, as well as changes in gene

24

regulation and transcription factor production (Waetzig and Herdegen, 2005;Yu *et al.,* 2008).

An ideal tissue for studying neuroplasticity in the peripheral nervous system must be easy for the researcher to access, stain, and observe in addition to being relatively simple while still being highly innervated. For instance, the cornea is clear, is very easy for researchers to access even in live specimens, is the most innervated tissue in the body and has a relatively simple structure (Harris and Purves, 1989; Marfurt and Ellis, 1993; Yu and Rosenblatt, 2007). Additionally, a number of researchers have been able to examine nerve regeneration in the cornea using ex vivo techniques (Harris and Purves, 1989; Yu and Rosenblatt, 2007; Yu *et al.*, 2008). Therefore, the cornea serves as a useful model for this study.

3. Aims of Research

The objective of this study is to establish a methodology that allows for the characterisation of neuroplasticity in the cornea of mice following a wounding event and to demonstrate that this model can be used to examine the interactions between nerves and immune cells during wound healing and inflammation in the context of studying hyperalgesia in the cornea.

It is hypothesized that it is possible to quantify neuroplasticity in the cornea by examining changes in the density and organisation of the nerves and nerve branching with

confocal microscopy combined with proprietary quantification software designed for this work. In order to test this hypothesis this study will first attempt to quantify the density of nerve branching in the cornea with an objective measurement that has known margins of error. If this is possible it is then hypothesized that chemical injury to cornea produces inflammation, characterised in this work as a statistically significant increase in neutrophils and macrophages, that results in the remodelling of nerves. It is hypothesized that there will be a reduction in the density of nerves immediately following the wounding event. If neuroplasticity is observable in the cornea, as suggested by the work of previous studies, the density of nerves is then hypothesized to increase over time, but to not reach the initial density of uninjured corneas within 48 hours.

This work proposes that by studying changes in nerve density and organisation following injury it is possible to characterise neuroplasticity during a wounding event in the cornea of mice. The significance of this research is to gain better understanding of the changes in nerve structure during an inflammatory response to an injury and the link this may have with the onset of hyperalgesia in the cornea. Furthermore, the quantification measurement and method, as well as the related proprietary software presented in this work is novel, and is presented as an improvement on the methods used to quantify nerve density in previous studies.

II. MATERIALS AND METHODS

1. Animals

Experiments were performed using female BALB/c mice (20-25 g; Charles Rivers, QC, Canada). The animals were housed in controlled conditions with a 12 hour light/dark cycle and unrestricted access to food and water. All experiments were conducted in accordance with the standards and procedures of the Canadian Council on Animal Care and the Dalhousie University Animal Care Committee.

2. Experimental Protocol

Injury Model

Adult (20-25g) female BALB/c mice were deeply anaesthetized with isoflourane (5%) in 100% oxygen. A toe pinch was performed to insure deep anesthesia before the right cornea of each mouse was cauterized at the 8'o'clock position with a silver nitrate applicator stick (75w/w% silver nitrate, 25%w/w potassium nitrate; Graham-Field Inc, Hauppauge, NY). Contact between the cornea and the applicator was maintained for 2 seconds, producing a discrete 1 mm diameter circular lesion that is greyish white in colour. Immediately after the injury the cauterized eye was rinsed with saline until the grey-white colour was no longer observed. The animal was then returned to its cage to recover. Animals recovered fully from anesthesia within minutes. The mice did not appear outwardly distressed, but did hold the cauterized eye slightly closed for up to three hours. This injury model is based upon the use of chemical cauterization with silver

nitrate to study tissue sensitisation following acute inflammation in adult Sprague-Dawley rats (Wenk and Honda, 2003).

In order to account for damage done to the cornea by simply placing an applicator in direct contact with the cornea, a sham operation was performed on three mice. These animals were treated with applicator sticks that did not have the silver nitrate/ potassium nitrate mixture. Other than this slight deviation from the procedure discussed previously, these control mice were subjected to identical treatment as their cauterized counterparts. A completely non-injured control is provided by the contralateral eye of each mouse in order to determine if the neurons of the cornea experience more neuroplasticity following an injury.

Experimental Groups

Allocation of mice was performed following a protocol modified from that used for rats by Wenk and Honda (2003). Briefly, animals were sacrificed with an intraperitoneal injection of 0.2 millilitres (mL) of euthanyl (sodium pentobartibal, 250mg/Kg, SOURCE) and confirmed by toe pinch and heart rate monitoring. Upon confirmation of death, the top (12 'o'clock position) of the cornea was marked with a needle tip and the whole eye removed with forceps. The injured and contralateral non-cauterized corneas were removed from 6 adult BALB/c mice at each of the following time points, after their right corneas were subjected to either cauterization with silver nitrate or a sham operation : 0,24,48 hours (Wenk and Honda, 2003). The mice were

divided up into experimental groups based upon the staining protocol to be performed (Table 1).

Table 1. Division of Experimental Animals by Treatment. Each group has a sample size of 3. H&E= Haematoxylin and Eosin stain. The immunohistochemical stain is a primary antibody stain for neuronal class III β Tubulin paired with a secondary antibody that is conjugated to Cy3, a red fluorescing molecule.

Group (n=3)	Treatment	Staining Protocol	Time Between Injury and Sacrificing of Animal (Hours)
Group 1	Control 1	H&E	0
Group 2	Control 2	Immunohistochemical	0
Group 3	Silver nitrate applied to right cornea	H&E	0
Group 4	Silver nitrate applied to right cornea	Immunohistochemical	0
Group 5	Silver nitrate applied to right cornea	H&E	24
Group 6	Silver nitrate applied to right cornea	Immunohistochemical	24
Group 7	Silver nitrate applied to right cornea	H&E	48
Group 8	Silver nitrate applied to right cornea	Immunohistochemical	48

Wholemount Examination of the Cornea with Immunohistochemisty

In order to investigate changes in the innervation of the cornea, wholemounts of both, cauterized and non-cauterized, corneas from the mice in groups 2, 4, 6 and 8 (Table 1) were prepared and analysed following a modified version of the protocol used by Yu and Rosenblatt (2007). Briefly, eyes were enucleated and fixed in 4% paraformaldehyde (Fisher Scientific, New Jersey, USA.) overnight at 4^oC (Celsius) then the corneas were removed under a dissection microscope (Carl Zeiss Microscopy, LLC, United States) and washed (2x15 minutes) in 0.1 Molar (M) phosphate buffered saline (PBS; Sigma-Aldrich, Saint Louis MO, USA) . The corneas were then permeabilized and blocked in freshly made 1% Bovine Serum Albumin (Sigma-Aldrich, Saint Louis MO, USA) and 0.2% Triton X-100 [4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol] (Sigma LifeScience, Saint Louis MO, USA) in PBS for 2 hours at room temperature (Yu and Rosenblatt, 2007).

Following blocking and permeablization the corneas were incubated in a 1:200 dilution of mouse anti-rat primary antibody stain for neuronal class III β Tubulin (Covanence, Montreal, CAN.) at 4° C overnight After washing in PBS (3x15 minutes) the corneas were placed in 1:500 diluted goat anti-mouse immunoglobulin G (IgG) secondary antibody conjugated to Cy3 (Jackson ImmunoResearch Inc, PA, USA) in the dark at 4° Celsius overnight. Following incubation the cells were rinsed in PBS (3x15 minutes). Relaxing cuts were then made in each cornea under a dissecting microscope before the corneas were mounted in mounting medium (Richard Allan Scientific, Thermoscientific, Kalamazoo, MI, USA), coverslipped, and then imaged with a confocal microscope (Zeiss

LSM 510 Laser Scanning Upright Microscope [Imager Z1 100], Carl Zeiss Microscopy, LLC, United States) equipped with software, a lamp (XBO 75W Lamp), and a computer (Intel Core 2 Duo CPU 3 GHz, Intel, USA) and ZEN 2009 imaging software (Carl Zeiss Microscopy, LLC, United States) and AxioCam microscope camera (Carl Zeiss Microscopy, LLC, United States). All confocal images were obtained using a 543nm HeNe laser and a 10x objective (EC Plan NEOFLUAR 10x/0.30 M27, Carl Zeiss Microscopy, LLC, United States).

Histopathology

Corneas from the mice in experimental groups 1, 3, 5 and 7 (n=3; See Table 1) were processed using standard Hematoxylin and Eosin (H&E) protocol and examined for the extent of immune cell infiltration, a common measure of inflammation, following a protocol similar to that used by Wenk and Honda (2003). This stain is commonly used to explore tissue histology, and is based on the different staining properties of H&E (Wenk and Honda, 2003). Haematoxylin is a basic dye that stains acidic structures, such as the DNA and ribosomes, and appears a purple-ish blue colour. Eosin is a basic dye that is known to stain basic structures pink, such as the cytosol, intracellular membranes and extracellular fibers. Briefly, whole eyes were immersion fixed in 4% paraformaldehyde (Fisher Scientific, New Jersey, USA.) at 4ºC overnight, stored in 15% and then 30% sucrose in 0.1 Mol/Liter PBS (Sigma-Aldrich, Saint Louis MO, USA) until they were embedded in optimum temperature cutting medium (Sakura Tissue-Tek, Torrance, CA)

31

and sectioned on the cryostat (CM1850, Leica, Ontario, Canada). Cross sections 5 μm thick were mounted in sets of 5 sections on positively charged glass slides (Fisherbrand superfrost® Plus microscope slides, USA), rinsed in tap water, submerged in Mayers Haematoxylin (Sigma Diagnostics, St. Louis, MO., USA) for 5 minutes, rinsed in tap water for 5 minutes, then dipped in a solution of 1% acid alcohol (70 absolute ethanol:30 distilled water:1 HCl, all from Fisher Scientific, New Jersey, USA.) rinsed in Scott's Tapwater Substitute (3.5 g Sodium bicarbonate, Sigma-Aldrich, Saint Louis MO, USA; 20.0g Magnesium Sulphate Sigma-Aldrich, Saint Louis MO, USA in 1 liter of tap water) for 5 minutes, dipped in 95% Ethanol (Commercial alcohols international, Ontario, Canada), followed by Y-Eosin (Sigma-Aldrich, Saint Louis MO, USA), before being rinsed in tap water for 5 minutes and cleared in 100% ethanol (Commercial alcohols international, Ontario, Canada) and xylene (Sigma-Aldrich, Saint Louis MO, USA). Mounting media (Cytoseal, Richard Allan Scientific, Thermoscientific, Kalamazoo, MI, USA.) was the applied and the slide coverslipped (VWR Microcover glass, USA).

3. Data Analysis

Evaluation of Immune Response Following Injury

A common measure of immune response in the cornea is the determination of immune cell infiltration over the time-course of the experiment (Wenk and Honda, 2003). To determine the extent of immune cell infiltration, H&E stained cross sections were

examined using a Nikon Digital DXml200F microscope camera at 2.5x and 20x

magnification. For each mouse, 4 sections were randomly chosen from those of the

control cornea and 4 sections from lesion containing sites of the cauterized cornea. These

sections were examined for the presence of immune cells. Neutrophils are easily

identified in H&E stained sections by their darkly staining multi-lobed nucleus and the

grainy appearance of their cytoplasm (Wenk and Honda, 2003), while macrophages have

darkly staining nuclei, and are large and irregularly shaped. The cells were counted for

the whole section and the mean number of cells per section was used to compare immune

cell infiltration in the cauterized relative to the contralateral control cornea for each

mouse. Cell count comparisons were made with one way analysis of variance (ANOVA)

tests and unpaired two sample t-tests, with statistical significance defined as $P<0.05$. All

data is reported as group mean±standard deviation.

Image Processing

Images taken from a confocal microscope, defined here as the source images,

undergo a three step image adjustment process (Figure 3.) using Adobe Photoshop

(Adobe Photoshop CS5, Adobe). First, a source image was inspected by the user: if there

were regions of the image where the local brightness was lower than in the majority of the

image (i.e.: due to unevenness in staining or to geometric deformity), the area was

manually selected using the Magnetic Lasso tool and brightened using Adobe

Photoshop's built-in Brightness/Contrast tool, such that the overall image brightness was more uniform at the expense of more apparent noise in the affected region (Oppenheim and Schafer, 2010). Second, the image was again inspected by the user and if the major nerve branches in the image were unclear, a copy of the image was processed with a high-pass filter and then superimposed onto an unfiltered copy of the image, creating a third image whose rapidly changing spatial features - noise and edges (nerves and nerve branches), but not background - were emphasized (Oppenheim and Willsky, 1997). Third, the image, whether high-pass filtered or not, was passed through a Nearest-Neighbor Median filter: this process removed confocal imaging artifacts (single, maximum intensity pixels) and reduced noise while preserving sharp edges (nerves) in the image (Nodes and Gallagher, 1982). An image resulting from the third step was defined as a preprocessed image.
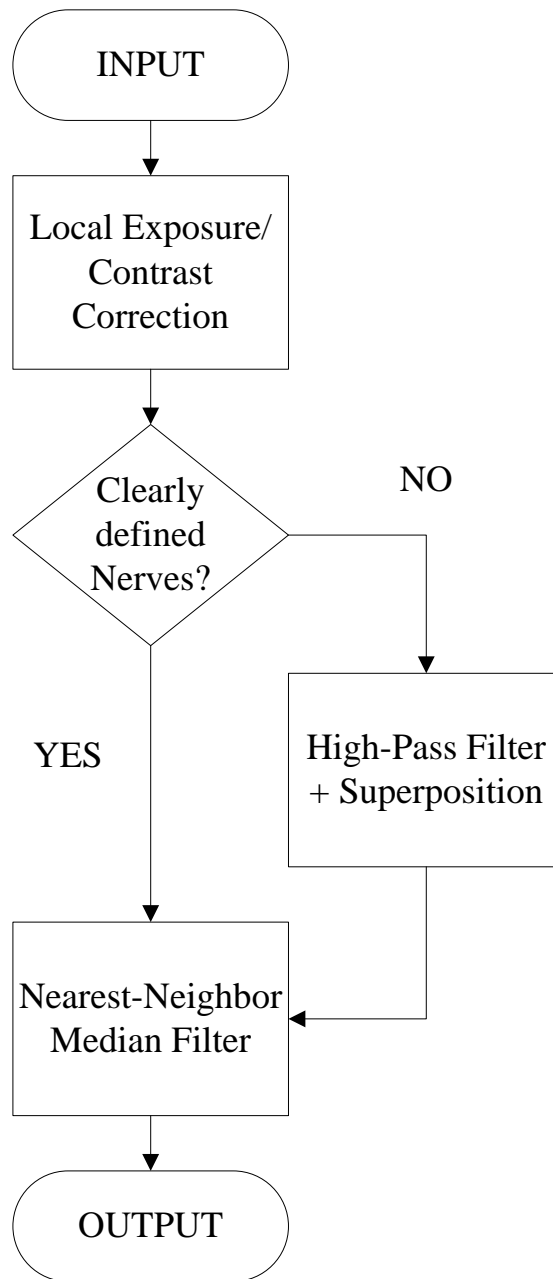
Figure 3. Flow Chart Depicting the Three Step Image Adjustment Process. Input is a source image taken by a confocal microscope. Output is the adjusted image that is to be quantified.

The analysis of the preprocessed images was performed using a proprietary software designed for this work (herein referred to as the program) and implemented in MathWorks MATLAB (R2010b), as an alteration and an enhancement to the widely used manual protocol developed by Yu and Rosenblatt (2007). The structure of the program is summarized in Figure 4. Each preprocessed image was given to the program as input. For each image, the user selected a region of the image to be excluded from the analysis (defined as the excluded region) in the case of edge-of-cornea imaging or the presence of otherwise nonviable pixels. The user then specified how many regions of interest (ROI) existed in the image: one if the image was a healthy control cornea, two if the image was of an injured cornea and contained regions both inside and outside of the wound boundary.

The user then guided the software in selecting pixels that corresponded to the desired nerve type. The pixel selected by the user and all other pixels in the image with an intensity (color value) within a set threshold value ($\pm$ 32 units by default) were automatically located and recorded internally, thus generating a ROI bit mask (an array of ones and zeros, ones representing valid pixel locations and zeros representing invalid locations) for that nerve type.

The ROI bit masks were then morphologically closed by the program using a 3x3 square connectivity matrix to ensure that nerve contiguity was preserved and that insufficiently connected areas were rejected as noise. Once the user was satisfied with the final bit masks generated by the program, the number of pixels (and therefore area) in

36

each region were then counted by the program. The nerve density was then computed as

the ratio of the number of pixels of the nerve type to the total number of unexcluded
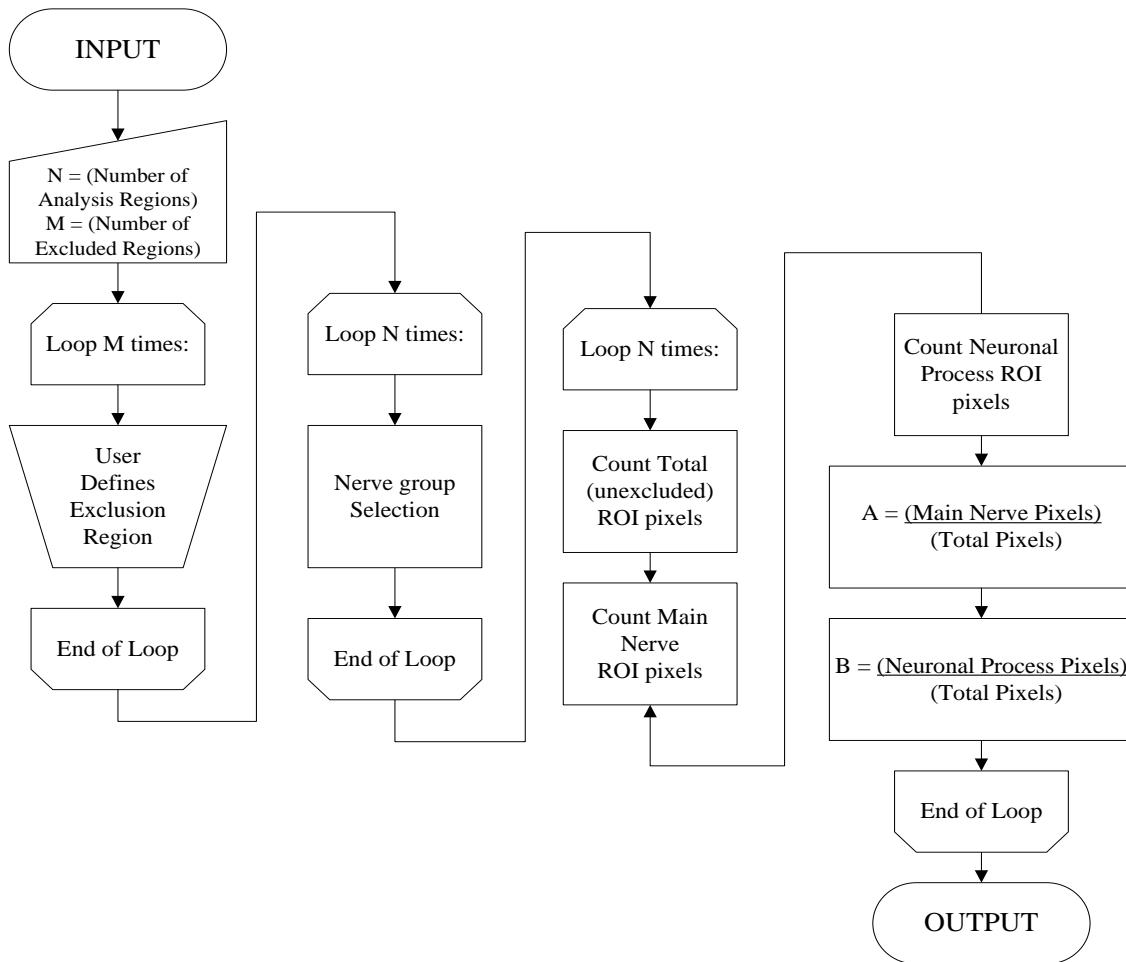
pixels in that ROI.

Figure 4. Flow Chart Depicting the Conceptual Design of the Proprietary Software Used
to Quantify Innervation of the Cornea. Input is a preprocessed image, as defined
previously. The user manually defines the number of included (N) and excluded
(M) regions and their boundaries within the image being analysed (left half of
Figure) before the program counts the number of pixels corresponding to each
nerve type, as defined by the user. The nerve density was then computed as the ratio
of the number of pixels of the nerve type to the total number of unexcluded pixels
in that ROI, with output A corresponding to the density of main nerves in the image
and output B corresponding to the density of neuronal processes in the image.

38

Statistical Analysis of Nerve Density

The density of major nerves and neuronal processes were calculated and analysed using Microsoft Excel 2007 (Microsoft, Washington, USA.). First, the number of pixels representing the area covered by the desired nerve type was converted to an area in mm^2 using the pixel to μm^2 conversion factor provided by the confocal microscope and converting that to mm^2. This was repeated for the total number of pixels in the region of interest the desired nerve group resided in. Additionally, a proportion of area covered by the desired nerve type (in mm^2) per total areal of the region of interest (in mm^2) was calculated. This proportion of areas is a numerical expression of the density of nerves in that area of the cornea similar to that shown in the literature (Yu *et al.,*2008; Yu and Rosenblat, 2007). All densities are reported as the experimental group mean ± standard error.

A one way analysis of variance (ANOVA) tests, with statistical significance defined as P<0.05 was used to analyse the difference between the mean densities of major nerves and neuronal processes in the corneas of the control mice, the corneas of the contralateral control eyes of experimental mice, and the area of the corneas outside of the wound boundary in the injured eyes of experimental mice. The data was then tested for significance with unpaired two sample t-tests, or in the case of comparisons made within the same animal (like in the case of comparing the density of nerves in the wound boundary to the density of nerves outside of the wound boundary) a paired two sample t-test was used. In all cases significance was considered at P<0.05.

III. RESULTS

1. Validation of Intergroup Comparisons

The density of major nerves and nerve processes in the corneas from control mice (n=6; no silver nitrate), the contralateral control eyes (n=9; naïve eyes) and the region of the cornea outside of the wound boundary in injured eyes (n=9) of mice subjected to chemical cauterization with silver nitrate were analysed for significant differences between their means using a one way ANOVA test (Figure 5 and Figure 6). The control cornea has a mean density of major nerves of 0.1284 ± 0.0373 mm^2/mm^2 and a mean density of neuron processes is 0.2506 ± 0.0498 mm^2/mm^2 while the corneas of the uninjured (contralateral) eye of the mice subjected to silver nitrate had a density of 0.0808 ± 0.0412 mm^2/mm^2 for major nerves and 0.3105 ± 0.0487 mm^2/mm^2 for neuronal process density. The area of the silver nitrate treated corneas that was outside of the edge of the wound had a density of major nerves of 0.0634 ± 0.0548 mm^2/mm^2 and a neuronal process density of 0.3204 ± 0.0473 mm^2/mm^2 .The P value for the density of major nerves and neuronal processes were 0.67 and 0.53 respectfully, which is higher than 0.05, indicating that within a 95% confidence interval the mean nerve density did not differ between the three aforementioned groups for both major nerves and nerve processes.
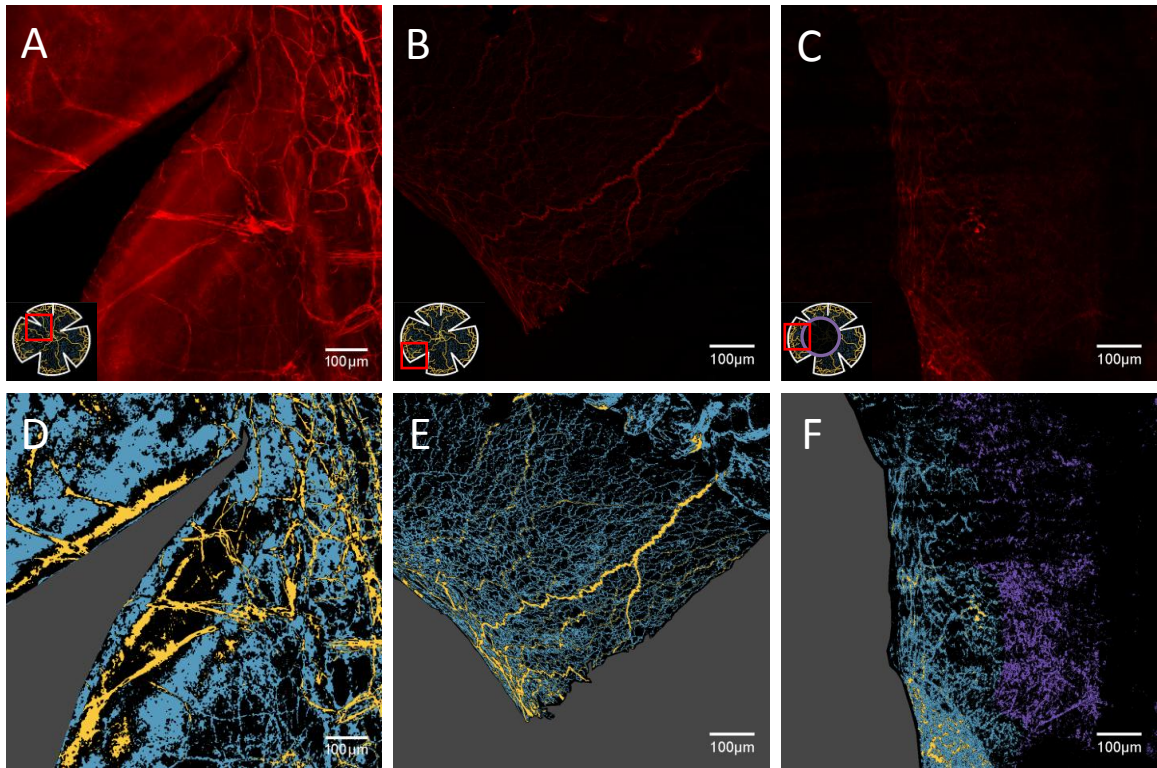
Figure 5. Confocal images of a cornea from a control mouse (A and D), contralateral control eye (B and E) and injured eye (C and F) of a mouse subjected to chemical cauterization with silver nitrate before (A, B, and C) and after (D,E, and F) image processing and analysis. Whole mounted corneas were stained with pan-neuronal class III β-tubulin primary antibody, followed by Cy3 conjugated secondary antibody. Representative images from each sample group show that the density of major nerves (shown in yellow in the processed images) and neuronal processes (blue) have similar densities (P>0.05). Grey portions of the processed images signify areas that were excluded from analysis. Inlaid diagrams in A, B and C are for orientation within the cornea in question

2. Evaluation of the Injury Model

A graphical representation of the changes in nerve density of the cornea following injury by chemical cauterization with silver nitrate is shown in Figure 6. In all experimental groups, each with a sample size of 3 mice, there was a statistically significant decrease (P<0.001) in the density of major nerves and nerve processes following a wounding event (Figure 6). Statistical significance was calculated using a unpaired two sample t-test in which the mean nerve density of the area of the cornea outside the wound boundary was significantly greater than the mean nerve density of the area of the cornea inside of the wound boundary point if P<0.05. These conditions were met for the experimental mice that were sacrificed 0, 24 and 48 hours after injury (Figure 6). Representative images of the localisation of nerves and nerve processes in an uninjured and injured cornea immediately after injury are shown in Figure 7 before (Figure 7,A) and after (Figure 7,B) image processing.
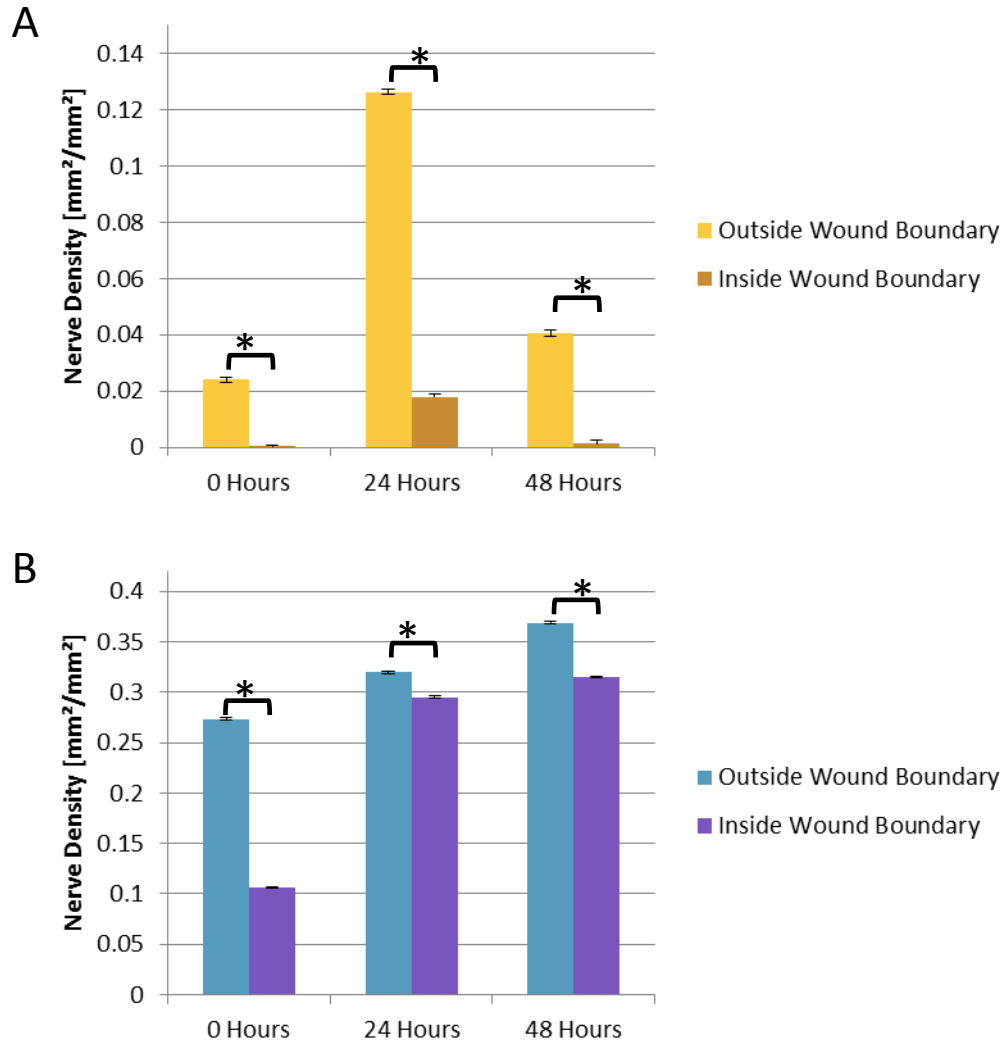
Figure 6. Nerve density significantly decreases after chemical cauterization with silver

nitrate. The mean density of major nerves (A) residing in the area of the cornea

within the boundary of the wound significantly decreases following wounding by

chemical cauterization with silver nitrate, as does the mean density of nerve

projections (B). This decrease is significant over a period of 48 hours. Statistical

significance ($P<0.05$) by paired two sample t-test is signified by an asterisks

symbol.
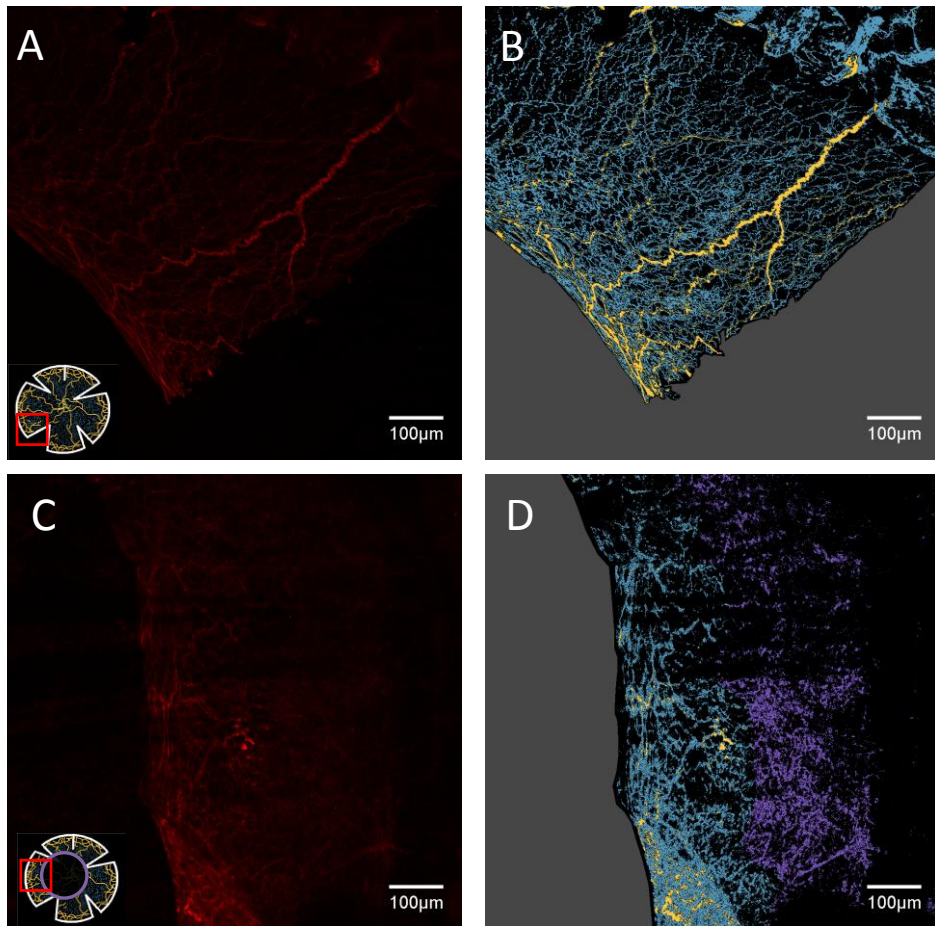
Figure 7. Localization of sensory innervation in mouse cornea wholemounts of corneas from uninjured control animals (A and B) or those subjected to treatment by silver nitrate (C and D). Whole mounted corneas stained with a pan-neuronal class III β-tubulin primary antibody, and visualized with Cy3 conjugated secondary antibody were imaged using a confocal microscope. Representative images from the control treatment group (A) and the corneas obtained immediately after silver nitrate treatment (C) show that the density and organisation of nerves in the wound area is significantly different to that of an unwounded eye.

3. Changes in the Density of Major Nerves and Nerve Processes Over Time

Over the course of 48 hours the average density of major nerves in the regions of the cornea inside the boundary of a circular wound caused by cauterization with silver nitrate increased from 0.00000 ± 0.00100 mm^2/mm^2 to a maximum value of 0.01801 ± 0.00080 mm^2/mm^2 after 24 hours, and then decreased to an average density of 0.00140 ± 0.00081 mm^2/mm^2 48 hours after the cornea was damaged (Figure 8). These results were determined to be statistically significant (P<0.0001). The density of neuronal processes within the boundary of the wound increased from 0.1063 ± 0.0008 mm^2/mm^2 at time zero to 0.31489 ± 0.00130 mm^2/mm^2 after 48 hours (Figure 8). This increase by 28.6% was found to be statistically significant by multiple unpaired two sample t-tests (P<0.0001) (Figure 8).

4. Evaluation of Immune Cell Infiltration into the Corneal Stroma Over Time

The average number of macrophages and neutrophils in the eye injured with silver nitrate at time 0 was 5.23 ± 2.83, while the uninjured contralateral eye had 8.70 ± 4.88 macrophages and neutrophils (See Figure 9,A). It was determined that there is no significant difference between the number of immune cells (macrophages and neutrophils) found in the stroma of the injured cornea and the uninjured contralateral eye (P>0.05 when analyzed by one way ANOVA followed by two sample t test;Figure 9,A). Twenty-four hours after the wounding event there was a sharp increase from the 7.5 ± 4.15 leukocytes and macrophages found in the cauterized cornea at 0 hours to an average of 205 ± 39 immune cells (P<0.05, Figure 9, A) The average number of neutrophils and

macrophages observed in the injured cornea 48 hours after injury was found to be 110±41

immune cells, which is significantly lower than the number of immune cells observed in

the injured corneas of mice after 24 hours (P<0.05 Figure 9A).
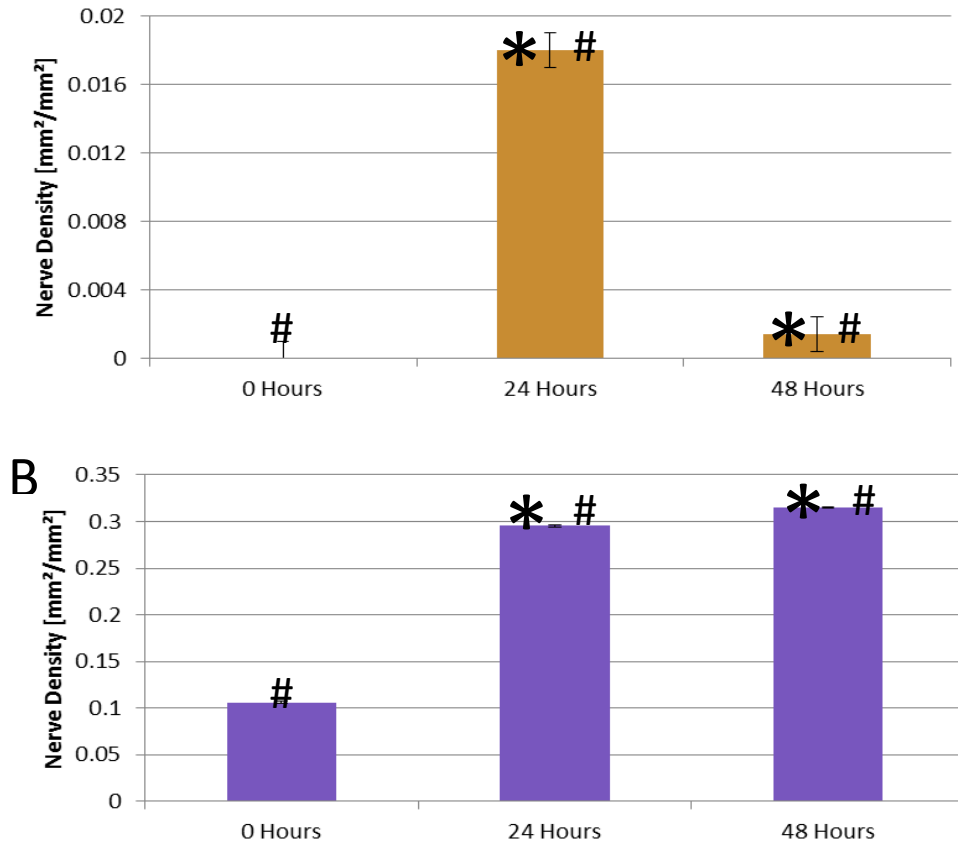
Figure 8. Mean nerve density in cornea at 0, 24 and 48 hrs after injury with silver nitrate

of main nerves (A) and neuronal extensions (B). Corneas were treated with

Silver Nitrate. # Symbolizes significantly less than the mean density of the

uninjured contralateral cornea of the same mouse, paired t-test p<0.05. *

Symbolises significantly (P<0.05) larger than the mean density of the preceding

experimental group by multiple two sample t tests.
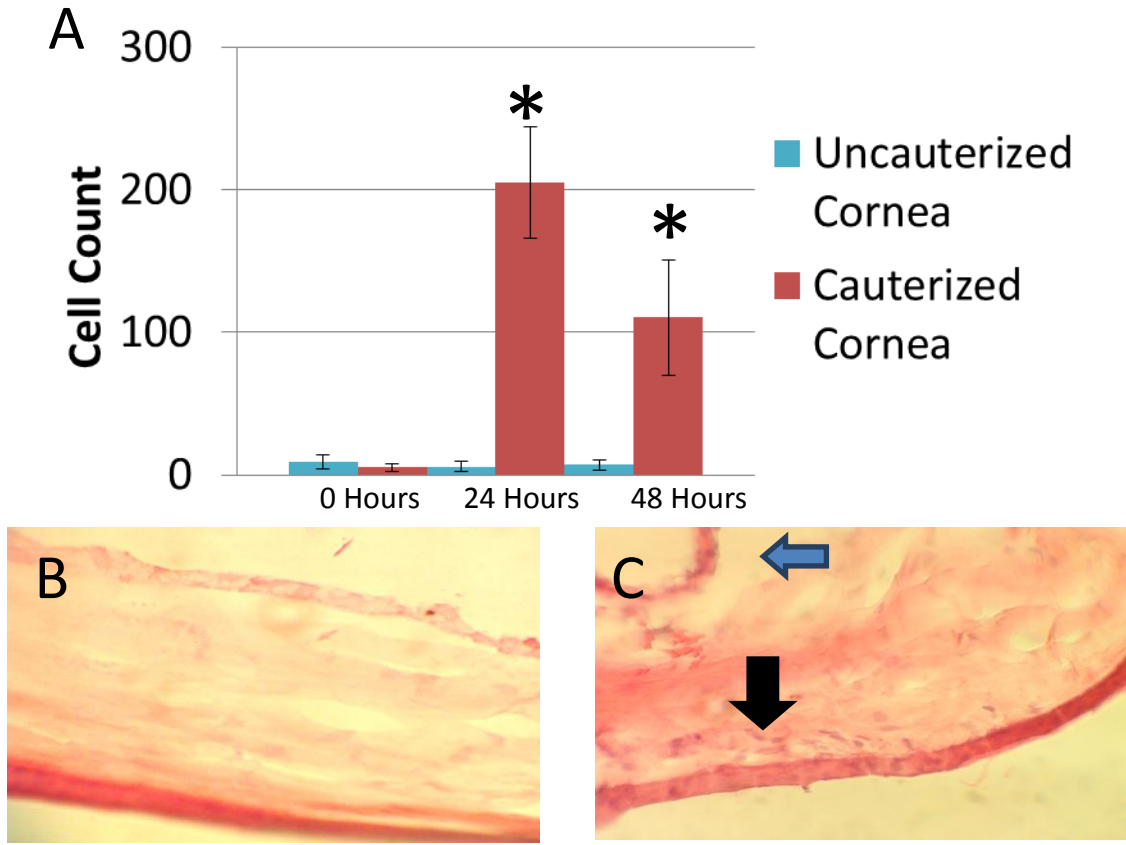
Figure 9. (A) The average immune cell counts from uncauterized and cauterized corneas at 0, 24 and 48hrs time points. (B&C)H&E histological staining of (B) non-injured cornea with no evidence of immune cell infiltration and (C) cauterized cornea with immune cell infiltration. Asteriks denote time points at which mean sectonal immune cell count for cauterized corneas is significantly greater than that of non-cauterized contralateral corneas. Asteriks also indicate that mean cell count for cauterized corneas at that time point is significantly greater than that of the naïve control group mice (data not shown).

P <0.001; macrophages (Black arrow) and stromal swelling (blue arrow).

IV. DISCUSSION

The role of neuroplasticity following a wound to an epithelium such as the cornea is of great importance to vision science, as it may help shape future medical approaches to common eye injuries in such a way that chronic eye pain and even blindness can be avoided (Yu and Rosenblatt, 2007; Yu *et al. ,* 2008; Belmonte *et al.,* 2004; DelMonte and Kim, 2011). The present study suggests that *Mus musculus* is a viable model for the study of changes in nerve morphology and density that are correlated with neuroplasticity. Furthermore, the work presented here suggests a means to better quantify neuroplasticity through an objective measurement, nerve density as a proportion of the area covered by nerves and the total area of the cornea, which has measurable margins of error, and is also investigated and reported on in this work. Additionally, the present study suggests further evidence that chemical injury to the cornea produces inflammation that results in the remodelling of nerves.

The majority of work looking into the innervation and immunity of the cornea has been done in humans (Navarro *et al.,* 2007; Waetzig and Herdegen, 2005; Lambiase *et al.*, 2000; Akpek and Gottsch, 2003) and rats (Wenk and Honda,2003; Lambiase *et al.*, 2000). There has been some preliminary work using mice to model innervations in the cornea (Harris and Purves, 1989; Yu and Rosenblatt, 2007; Yu *et al.,* 2008) however these models do not try to account for the impact of the immune response on changes in corneal nerves after the cornea has been damaged.

Troublingly, previous work on this topic often reports their results as pictomicrographs from a microscope, or manually traced approximations of nerve paths within the cornea (Harris and Purves, 1989; Belmonte *et al.,* 2004). Some groups took this one step further and quantified these drawings by calculating the approximate length of nerves within one field of view of a cornea after manually tracing them (Yu and Rosenblatt, 2007; Yu *et al.,* 2008). However, in this era of digital imaging technology it is not inconceivable that the area covered by nerves in an image of the cornea can be calculated automatically, removing the errors that would added by manually tracing the nerve paths (See Figure 4 and Figure 5). The method used to quantify nerve density used in this report does this exact automatic calculation, and produces similar results (See Figure 6) to those seen in the literature on the topic, such as the work by Yu *et al.*(2007, 2009). This suggests that the more robust method used here is comparable to the more error prone methods seen in previous published work, and may therefore be seen as a more robust alternative for future studies.

Now that it has been established in this work, and in the work of others, that neuroplasticity can be investigated by examination of the quantifiable changes in the density of nerves in the cornea, it must be demonstrated that there is no significant difference between the innervation, measured as mean nerve density of main nerves and their projections, of the corneas on both eyes of the same mouse or corneas between different mice, as this must be true for comparisons to hold any statistical significance. By investigating if this measurement is consistent from eye to eye and from mouse to mouse this work can demonstrate the robustness of the automatic quantification technique

49

presented in this work. It was found that the mean nerve density of both the main nerves and their projections are not significantly different between different animals and between different eyes of the same animal (See Figure 5 and Section III.1). In the context of this study, this means that comparisons of the nerve density within the boundary of a wound to the area outside of the wound boundary on the same eye carry the same statistical weight as comparisons to the uninjured eye and to control animals that were not injured at all. However, it should be taken into consideration that the sample sizes used in this study were not very large, such that it is preferable that larger sample sizes are used to further test the assumptions that the controls are valid.

The wound being investigated here is chemical cauterization by silver nitrate. This injury model has been extensively characterised and shown to induce inflammation and hyperalgesia in rats (Wenk and Honda, 2003). The results presented in this work suggest that this injury model affects mice in a similar manner, as the silver nitrate creates a reproducible circular wound similar to that reported in rats, and the patterns of immune cell infiltration following the wounding event appear to be similar between rats and mice (See Figure 9; Wenk and Honda, 2003). It was determined that there was a peak of immune cell infiltration in the corneal stroma 24 hours after injury, and that after this time the immune cell count decreased slightly (Figure 9). These results are consistent with literature (Wenk and Honda, 2003), suggesting that silver nitrate induces inflammatory responses in the murine cornea similar to those observed in the cornea of rats.

Now that it is has been established that it is possible to observe and investigate inflammation (measured by examination of the amount of macrophages and neutrophils infiltrating the corneal stroma over a time course) and innervation of the cornea (measured by the density of major nerves and neuronal processes) in mice using silver nitrate as the injury model this study proceeded to take the work of Wenk and Honda one step further by investigating the effects of chemical cauterization with silver nitrate on the density of nerves in the cornea. Wholemounts of the cauterized and injury free eye of experimental mice were stained with βIII-tubulin variant specific to neurons, imaged and were analysed for the density of the nerves in different areas of the cornea.(See Figure 5; Yu and Rosenblatt, 2009). The results of this study indicate that cauterization with silver nitrate significantly reduces the density of both major nerves and thin neuronal projections in the area of the cornea that was cauterized relative to uninjured portions of the same eye (Figure 6 and 7). This significant reduction in nerve density is also observed in both major nerves and thin neuronal projections in the area of the cornea that was cauterized when compared to, nerve densities of the uncauterized eye on the same animal, or the densities of nerves and nerve projections in control animals (See Figure 6 and 7). This suggests that chemical injury reduces the density of nerves in the cornea, as hypothesized. Additionally, the cauterization procedure left the area of the cornea outside of the outer boundary of the cauterization wound with nerve and neuronal projection density of both nerve types similar to the densities observed in both control eyes on the same animal and the corneas of control animals (See Figure 5,6 and 7). This suggests that the controls used in this study were appropriate and allow for statistically robust

statements to be made about the impact of chemical injury on the density of nerves in the cornea.

Chemical cauterization of the murine cornea resulted in a significant decrease in the density of both major nerves and neuronal extensions, as expected (Figure 6 and Figure 7). Following this large drop in nerve density immediately following injury, the cornea appeared to recover over time, as there was a significant increase in both main nerve and nerve extension density of the cornea when the mice were allowed to recover for 24 hours (Figure 8), suggesting that neuroplasticity is observed in the cornea, as hypothesized in this study. There was also a significant increase (28.6%, P<0.05) in the density of neuronal processes over 48 hours, but this was accompanied by a significant decrease in main nerve density from 24 to 48 hours (Figure 8). This sudden decrease in major nerves may be due to the pruning of neurons that did not reach their target effector cells; however this is has not been observed in the cornea to the knowledge of the author at the time of writing this report. The possibility that pruning, which is often seen to quickly impact neuroplasticity in the central nervous system (McKay, 1997; Shihabuddin, Palmer, and Gage, 1999), may play a role in neuroplasticity in the cornea is an exciting possibility that deserves further investigation in future studies.

The increase in nerve density over time has previously been shown to be correlated to neuroplastic changes in the corneal nerves in mice that were bred to have yellow fluorescing protein (Yu and Rosenblatt, 2007; Yu et al., 2008). The results in this work and the work of others suggest that the density of nerves in the wounded portion of

the cornea starts to increase again after an injury, as the regrowth of neuron axons and dendrites reconnect the old neurons in old and possibly new ways that are definitive of neuroplasticity (Figure 8; Yu and Rosenblatt, 2007; Yu *et al.,* 2008, Navarro *et al.,* 2007). Additionally, the rate that nerve density is regenerated following injury follows the results obtained in the cornea of mice (Yu and Rosenblatt, 2008) and even in the spinal cords of monkeys (Belmonte *et al.,* 2004), suggesting that the murine model proposed in this work is appropriate for the study of neuroplasticity in the cornea. Unfortunately the mechanisms behind this neuroplastic changes are not well understood, despite plenty of research on the topic, suggesting that further research is needed (Navarro *et al.,* 2007).

As such, the work presented in this study opens up paths for future research to further investigate the possible relationship between neuroplasticity in the cornea following a wounding event and the infiltration of immune cells that is observed as part of the inflammatory response following injury in the cornea. It was determined that there was a peak of immune cell infiltration in the corneal stroma 24 hours after injury, and that after this time the immune cell count decreased slightly (Figure 9). These results are consistent with literature (Wenk and Honda, 2003) and appear on the same temporal scale as the initiation of changes nerve density (Figure 8 and 9). This temporal link should be investigated more closely in the future by adding experimental time points before 24 hours to those presented here. Furthermore, future work should include an investigation of the ability of compounds commonly secreted by macrophages and neutrophils, such as cytokines, to interact with and potentially moderate the activity of neurons in the cornea.. Furthermore, corneal nerves are known to secrete neurotransmitters that impact the

53

immune response in the cornea, so it would not be unsurprising if the reverse were also true (Akpek and Gottsch, 2003), inviting another avenue for future studies using the model presented here.

This work suggests that the mouse is a suitable model organism for the study of neuroplasticity and the processes of inflammation following an injury to the cornea and that it is possible to better quantify neuroplasticity in the murine model. Additionally, inflammation and neuroplasticity were both observed, as hypothesized. In addition to further studying the mechanisms behind these processes with the model presented here, it is suggested that the experiments conducted in this report be repeated with larger sample sizes such that more robust statistical analysis is possible.

It was determined that chemical injury to the cornea with silver nitrate immediately causes a significant decrease in the density of major nerves and their processes within the boundary of the wound, and that within 48 hours this injury produces inflammation followed by a significant increase in nerve density (Figures 5-9). It is important to note that the nerve density within the boundary of a wound never reaches the densities of nerves or nerve processes in uninjured corneas, as hypothesized (Figure 6 and 8). Interestingly, it was observed that the density of nerves and nerve processes in the wound increases while the infiltration of immune cells begins to decrease, suggesting that future study should investigate a possible link between the two processes.

Most notably, the novel automated quantification method used in this work is considered an improvement on the methods previously used in this field (Yu and

Rosenblatt, 2009). The methods previously used to investigate neuroplastic changes in the cornea were wrought with large possibilities for human error that are significantly reduced by using the computerised methods proposed in this study. Work needs to be done with better quantifying changes nerve branching over time, and the methods proposed in this research are a major step in that direction. It is also suggested that future studies consider using transgenic mice, automated nerve density calculating software like that presented here, or attempt to fit the nerve densities in literature to a model whose sampling distribution is already confined to [0,1] such that the sampling distribution for nerve densities might be able to be approximated by some kind of beta distribution for better statistical evaluation.

Future work can use the results of this study as a baseline for comparison of the effects of pharmacological compounds on nerve remodelling in the cornea. Additionally, this work has medical implications, as a better understanding of nerve remodelling in the cornea may allow for the development of methods that reduce or remove complications such as dry eye after operations such as LASIK surgery, and may be vital to promoting the healing of the cornea after accidental injury or disease, potentially saving the vision of numerous people who would have otherwise gone blind. Mastering neuroplasticity in the cornea may one day lead to an understanding of how to induce the same nerve remodelling in related structures, like the skin, which has many medical applications and may eventually even lead to the ability to induce nerve regeneration in the brain.

Furthermore, it is suggested that in the future this model should be used to study the relationship between a hyper sensitivity to pain, known as hyperalgesia, and the observable changes in nerve density and morphology following injury. If hyperalgesia in the cornea is linked to such neuroplastic changes it may be possible to then use this model to attempt modulating and treating this pain such that a new treatment method is proposed for those who suffer from neuropathic pain and hyperalgesia in the cornea. The investigation of the interactions between nerves and the immune cells during wound healing and inflammation in the context of studying hyperalgesia is the driving force of this study as increased understanding of such phenonena may result in clinical applications that make dry and blindness no longer likely symptoms of corneal damage.

REFERENCES

Akpek E, Gottsch J. 2003. Immune defense at the ocular surface. Eye. 12:949-956

Alberts B, Bray D, Hopkin K, Johnson A, Lewis J, Raff M, Roberts K, and Walter P. 2010. Essential cell biology. 3rd ed. New York. Garland Science.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P.2002. The Adaptive Immune System in Molecular Biology of the Cell, 4th ed. Garland Science: New York.

Altman J. 1962.Autoradiographic study of degenerative and regenerative proliferation of neuroglia cells with tritiated thymidine. Exp Neurol. 5:302-318.

Altman J.1962. Are new neurons formed in the brains of adult mammals? Science.135:1127-1128.

Altman J and Das G. 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 124:319-335.

Armentia M, Cabanes C, and Belmonte C.2000. Electrophysiological properties of identified trigeminal ganglion neurons innervating the cornea of the mouse. Neuroscience. 101(4):1109-1115

Belmonte C, Acosta M, and Gallar J.2004. Neural basis of sensation in intact and injured corneas. Experimental Eye Research. 78: 513–525

Belmonte C, Garcia-Hirschfeld J, and Gallar J. 1997. Neurobiology of ocular pain. Prog. Retin. Eye Res. 16, 117–156.

Burnett M and Zager E.2004. Pathophysiology of peripheral nerve injury: a brief review. Neurosurg Focus.16:1–7.

Cajal S.1894. The Croonian lecture: La fine structure des centres nerveux. Proc Roy Soc London. 55:444-467

Campbell W.2008.Evaluation and management of peripheral nerve injury. Clinical Neurophysiology.119(9):1951-1965.

Chao C, Golebiowski B, and Stapleton F. 2014. The role of corneal innervations in LASIK-induced neuropathic dry eye. The Ocular Surface. 12(1):32-45.

Chen R, Cohen L, Hallett M. 2002. Nervous system reorganization following

injury. Neuroscience; 111: 761–73

DelMonte D and Kim T. 2011. Anatomy and physiology of the cornea. J Cataract Refract Surg.; 37:588–598

de Felipe C and Belmonte C. 1999. c-Jun expression after axotomy of corneal trigeminal ganglion neurons is dependent on the site of injury. Eur. J. Neurosci. 11, 899–906.

Duraiyan J, Govindarajan R, Kaliyappan K, and Palanisamy M. 2012. Applications of immunohistochemistry. J Pharm Bioall Sci. 4, Suppl S2:307-9

Enciu A, Nicolescu M, Manole C, Mureşanu D, Popescu L, and Popescu B.

2011. Neuroregeneration in neurodegenerative disorders. BMC Neurol 11:75.

Harris L and Purves D. 1989. Rapid remodeling of sensory endings in the corneas of

living mice. The Journal of Neuroscience 9(6):2210-4.

Haughey N, Nath A, Chan S, Borchard A, Rao M, and Mattson M.2002. Disruption of

neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell

homeostasis, in models of Alzheimer's disease. J Neurochem . 83:1509-1524.

Jenkins W and Merzenich M. 1987. Reorganization of neocortical representations after

brain injury: a neurophysiological model of bases of recovery from stroke. Prog.

Brain Res. 71: 249-266.

Johansson B. 2007. Regeneration and plasticity in the brain and spinal cord. J Cereb

Blood Flow Metab.27:1417-1430.

Kaiser E. 2012. The innate immune system. Retrieved from

http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit4/innate/iflammation.html.

Ksander B, Paraskevi E. Kolovou P, Wilson B, Saab K, Guo Q, Ma J, McGuire S,

Gregory M, Vincent W, Perez V, Cruz-Guilloty F, Kao W, Call M, Tucker B, Zhan

Q, Murphy G, Lathrop K, Alt C, Mortensen L, Lin C, Zieske J, Frank M and Frank

N.2014. *ABCB5* is a limbal stem cell gene required for corneal development and

repair. Nature [Internet]. [revised 02 July 2014;cited 03 November

2014]nature.com:Nature. Available from

http://www.nature.com/nature/journal/vaop/ncurrent/full/nature13426.html.

Lambiase A, Rama P, Bonini S, Caprioglio G, and Aloe L.1998. Topical treatment with nerve growth factor for corneal neurotrophic ulcers. N Engl J Med 338:1174-80.

Lambiase A, Manni L, Bonini S, Rama P, Micera A, and Aloe L. 2000. Nerve growth factor promotes corneal healing: Structural, biochemical, and molecular analyses of rat and human corneas. Invest Ophthalmol Vis Sci 41(5):1063-9.

Marfurt C and Ellis L.1993. Immunohistochemical localization of tyrosine hydroxylase in corneal nerves. J Comp Neurol. 336:517–531.

McKay R. 1997.Stem cells in the central nervous system. Science.276:66-71.

McKinley P, Jenkins W, Smith, and Merzenich M. 1987. Age-dependent capacity for somatosensory cortex reorganization in chronic spinal cats. Dev. Brain Res. 31:136-139.

Morshead C, Reynolds B, Craig C, McBurney M, Staines W, Morassutti D, Weiss S, and van der Kooy D. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron. 13:1071-1082.

Moulin D, *et al.*2007. Pharmacological management of chronic neuropathic pain – consensus statement and guidelines from the Canadian Pain Society. Pain Res Manag. 12 (1): 13-21.

Müller L, Marfurt C, Kruse F, and Tervo T. 2003. Corneal nerves: structure, contents and function. Experimental Eye Research. 76:521-542.

National Institute of Neurological Disorders and Stroke (NINDS). National Institutes of Health, 28 Apr. 2014. "The Life and Death of a Neuron." Web. 6 Nov. 2014. <http://www.ninds.nih.gov/disorders/brain_basics/ninds_neuron.htm>.

Navarro X, Vivo M, and Valero-Cabre A. 2007. Neural plasticity after peripheral nerve injury and regeneration. Progress in Neurobiology.82:163–201

Niederkorn JY, Peeler JS, and Mellon J. 1989. Phagocytosis of particulate antigens by corneal epithelial cells stimulates interleukin-1 secretion and migration of Langerhans cells into the central cornea. Reg Immunol; 2: 83–90.

Nodes T and Gallagher N. 1982. Median Filters: Some Modifications and Their Properties. IEEE Transactions on Acoustics, Speech, and Signal Processing. ASSP-30(5):739-746.

Oppenheim A and Schaefer R. 2010. App. A Random Signals in *Discrete-Time Signal Processing Third Ed.* Pearson Higher Eduction Inc. Upper Saddle River. USA. 1050-1052.

Oppenheim A and Willsky A. 1997. Frequency-Selective Filters in *Signals and Systems Second Ed.* Pearson Higher Eduction Inc. Upper Saddle River. USA. 236-239.

Ramon Y and Cajal S. 1894.The Croonian lecture: La fine structure des centres nerveux. Proc Roy Soc London.55:444-467.

Reynolds B and Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science.255:1707-1710.

Shihabuddin L, Palmer T, and Gage F. 1999. The search for neural progenitor cells: prospects for the therapy of neurodegenerative disease. Mol Med Today.5:474-480.

Slonczewski J and Foster J.2011. Observing the microbial cell. In: Microbiology an evolving science. 2nd ed. New York. W.W Norton and Company.

Stepp M, Zieske J, Trinkaus-Randall V, Kyne B, Pal-Ghosh S, Tadvalkar G, and Pajoohesh-Ganji A.2014. Wounding the cornea to learn how it heals. Experimental Eye Research. 121:178-193.

Waetzig V and Herdegen T. 2005. MEKK1 controls neurite regrowth after experimental injury by balancing ERK1/2 and JNK2 signaling. Mol. Cell. Neurosci. 30:67–78.

Wenk H and Honda C. 2003. Silver nitrate cauterization: characterization of a new model of corneal inflammation and hyperalgesia in rat.Pain. 105(3):393-401.

Yu C, Zhang M, Matis K, Kim C, and Rosenblatt M. 2008. Vascular endothelial growth factor mediates corneal nerve repair. Invest Ophthalmol Vis Sci 49(9):3870-8.

Yu,C: Rosenblatt M. 2007. Transgenic Corneal Neuroflourescence in Mice: A New Model for In Vivo Investigation of Nerve Structure and Regeneration. Invest Ophthalmol Vis Sci.48:1535–15