Corneal neurotization maintains corneal epithelial integrity and restores nerve-derived peptides in a rat model of neurotrophic keratopathy

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto
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Abstract

PURPOSE: Investigate effect of corneal neurotization on epithelial integrity and restoration of nerve-derived peptides in denervated rat corneas.

METHODS: Three rat corneal conditions were assessed (n=45): denervation, denervation + neurotization, normal innervation. Corneal epithelial and stromal thicknesses were evaluated using Hematoxylin and Eosin staining. Corneal ulceration and perforation were assessed under a Wood's lamp and normal light, respectively. Immunohistochemistry and western blot were used to evaluate Substance P (SP) and calcitonin gene-related peptide (CGRP) presence.

RESULTS: Where significance is \( p<0.05 \), central epithelium was thinner in the denervated compared to the denervated + neurotized and normally innervated corneas. The denervated stroma was thinner than the normally innervated, but not the denervated + neurotized stroma. Neurotization protected the denervated cornea from ulceration and
perforation. Neurotization restored the denervated cornea with SP and CGRP, co-localized with axons innervating the cornea.

**CONCLUSION:** Corneal neurotization restores epithelial integrity and nerve-derived peptides in the denervated cornea.
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<th>Description</th>
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<tbody>
<tr>
<td>BCVA</td>
<td>Best corrected visual acuity</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CP</td>
<td>Common peroneal</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FNE</td>
<td>Free nerve endings/intraepithelial nerve terminals</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1 (IGF-1)</td>
</tr>
<tr>
<td>LP</td>
<td>Limbic plexus</td>
</tr>
<tr>
<td>LSC</td>
<td>Limbal stem cell</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NK</td>
<td>Neurotrophic Keratopathy</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin 3</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin 4/5</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PED</td>
<td>Persistent epithelial defect</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SBP</td>
<td>Subbasal plexus</td>
</tr>
<tr>
<td>SC</td>
<td>Schwann cell</td>
</tr>
<tr>
<td>SCcm</td>
<td>Schwann cell conditioned media</td>
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<tr>
<td>SCpm</td>
<td>Schwann cell pre-conditioned media</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEP</td>
<td>Subepithelial plexus</td>
</tr>
<tr>
<td>SN</td>
<td>stromal nerve trunks</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TAC</td>
<td>Transient amplifying cell</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanillinn type 1</td>
</tr>
<tr>
<td>V1</td>
<td>Ophthalmomaxillary nerve</td>
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CHAPTER 1  Introduction and Literature Review
1.1 Preamble

The cornea is the clear part of the eye through which you can see. Its transparency allows for transmission of light to the retina for visual processing, and consequently allows for normal vision. The tissue’s transparency is maintained via the dense, regular arrangements of cells and extracellular components in the corneal layers and via avascularity (DelMonte and Kim, 2011). The cornea is one of the most densely innervated tissues of the body (Rózsa and Beuerman, 1982), and its innervation is critical for maintenance of ocular surface health, transparency, and consequently vision. Corneal sensory nerves are responsible for the protective blinking and tearing reflexes that prevent corneal abrasion and expel foreign objects. These nerves provide the cornea with trophic support and survival signals for the corneal epithelium via release of nerve-derived peptides, that, at least in part, maintain and heal the corneal epithelium after injury (Müller et al., 2003).

The importance of corneal innervation is apparent in its absence. In absence of corneal sensory innervation and hence, sensation, patients develop Neurotrophic Keratopathy (NK). This disease is characterized by corneal epithelial breakdown, progressive corneal scarring, and can eventually result in vision loss. NK treatment in is focused primarily on ophthalmic management of symptoms primarily. However, these treatments fail to treat the underlying cause of NK (Reviewed by Sacchetti and Lambiase, 2014; Semeraro et al., 2014; Mastropasqua et al., 2017). Corneal neurotization is a surgical procedure that re-innervates the NK cornea by nerves directed to the cornea via nerve autografts. In patients, this procedure restores corneal sensation and innervation (Fung et al., 2018). However, because ~15% of treated corneas fail to acquire protective corneal sensation (Catapano et al., 2019), we have developed an animal model of NK and corneal neurotization to investigate the effects of the neurotization surgery and its mechanisms. In this model, corneal neurotization resulted in improved healing of the corneal epithelium after injury, as compared to the denervated NK cornea. Further studies are required to determine if corneal neurotization maintains epithelial integrity and restores the cornea with the nerve-derived mediators necessary to preserve corneal health.
1.2 Thesis organization

This thesis is organized in “paper format” using modified peer-reviewed content and unpublished data where indicated. Chapter 1 introduces corneal anatomy and physiology in the healthy cornea, neurotrophic keratopathy (NK) and current NK treatments, including corneal neurotization. Chapter 2 presents original research evaluating the effect of corneal neurotization on corneal epithelial and stromal thickness and on the presence of nerve-derived peptides in the denervated NK cornea. This chapter further compares the effect of topical treatments, nerve growth factor and Schwann cell conditioned media, on healing an epithelial injury in the denervated cornea with each other, and with the previously published healing effect of corneal neurotization (Catapano et al., 2018). Part of this chapter was reformatted from work published in the journal of IOVS (Catapano et al., 2018). This work investigates corneal ulceration and scarring in the denervated and neurotized corneas. In this chapter, the future directions specific to the presented results are discussed. Chapter 3 summarizes the results in a broader context of the existing literature and outlines ongoing and future directions.
1.3 Corneal Anatomy and Physiology

I - INTRODUCTION

The corneal structure informs corneal function. Corneal sensory innervation is critical in maintaining a clear and functional cornea. In this chapter, corneal anatomy and physiology are summarized and an emphasis is placed on corneal innervation and its influence on the surrounding structures.

II - CORNEAL ANATOMY

The cornea plays a critical role in vision. It protects against infection and injury by providing a barrier between the external environment and internal ocular contents (Figure 1-1). The cornea is responsible for the majority of the total refractory power of the eye, and consequently normal vision requires maintenance of corneal shape (Meek et al., 2003; Meek and Knupp, 2015). Clarity is achieved by there being no blood vessels or myelinated axons in addition to the layered arrangements of the corneal cells.

The cornea is comprised of five layers. The most anterior three layers are innervated by corneal sensory nerves and two posterior layers are not supplied by any innervation. From anterior to posterior these are the: i) corneal epithelium ii) Bowman’s layer iii) stroma iv) Descemet’s membrane v) endothelium (Figure 1-1B).
The corneal epithelium functions primarily as a protective barrier. In humans, this corneal layer is about 50 μm thick and is composed of four to six layers of non-keratinized stratified squamous epithelial cells. The two to three most superficial cell layers contain flattened nuclei. The deeper wing (suprabasal) epithelial cells are polyhedral and the basal epithelial cells are columnar in shape (Eghrari et al., 2015) (Figure 1-1C). Epithelial cells adhere at tight junctions via proteins such as ZO-1, JAM-A, E-cadherin, occludin and caludin-1 (Ban et al., 2003). The tight junctions between epithelial cells function to

**Figure 1-1 Corneal anatomy.** A. Outline of the anterior features of the globe with an emphasis on the cornea (boxed) in relation to other anatomical features (Navaratnam et al., 2015). B. A sagittal section of the cornea demonstrates the five corneal layers (Navaratnam et al., 2015). C. A sagittal section of the corneal epithelial cell layer (i).
create a watertight seal which assists in preventing pathogenic organisms from penetrating the cornea (Ban et al., 2003; DelMonte and Kim, 2011; Eghrari et al., 2015). Human cultured corneal epithelial cells also secrete pro-inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α (Zhang et al., 2003; Kumar et al., 2004; Zhang et al., 2005; Kumar et al., 2006), which are known to assist in immune regulation and protection against pathogens (Harder et al., 2000; Liu et al., 2014). In the basal corneal epithelium, the Langerhan cells are involved in the corneal immune response (Hamrah and Dana, 2007).

The superficial surface of the corneal epithelium is covered by apical microvilli and microplicae, a charged glycocalyx and a layer of tear film (Figure 1-1C). This 7 μm tear film is composed of mucinuous, aqueous and lipid layers, each contributing to the overall function of the cornea. The tear film “smooths out micro-irregularities” of the superficial anterior epithelial surface (DelMonte and Kim, 2011) and prevents desiccation, infection and friction from opening and closing of the eyelids during blinking (Perry, 2008). The lipid layer of the tear film is the most superficial layer and is secreted by the Meibomian glands at the rim of the eyelid in the tarsal plate (Perry, 2008; DelMonte and Kim, 2011; Eghrari et al., 2015). The aqueous layer is secreted primarily by the lacrimal glands located in the upper lateral region of the orbit, and partially by the accessory lacrimal glands in the conjunctiva. Tear secretion from the lacrimal gland is stimulated by afferent innervation from the trigeminal nerve and efferent innervation from parasympathetic and sympathetic innervation (Dartt, 2011). The aqueous secretions from these glands that form this layer of the tear film are isotonic and contain proteins such as lysozomes, lacritin and others (McKown et al., 2009). The deepest, mucous, layer of the tear film is secreted by conjunctival goblet cells. These cells are innervated by efferent sympathetic and parasympathetic nerves. Secretion of this layer, predominantly containing the polysaccharide MUC5AC, is stimulated by noxious stimulation of sensory nerves in the cornea (Dartt, 2011). The glycocalyx sits between the superficial tear film and the corneal epithelium to hydrate the corneal surface and protect it from infection (Dartt, 2011). It is composed of soluble and membrane-spanning polysaccharides, such as MUC1, MUC4,
and MUC16, as well as those secreted by the lacrimal gland, goblet cells, and the corneal squamous epithelium (Argüeso and Gipson, 2001; Gipson, 2004).

The lifespan of individual epithelial cells is approximately seven to ten days (Hanna et al., 1961; Eghrari et al., 2015), and complete cellular turnover is estimated to occur in about two weeks (Cenedella and Fleschner, 1990). The limbal epithelial stem cells (LSCs) are located at the limbus, the junction of the cornea and sclera. During normal cellular turnover, the LSCs proliferate, differentiate, and terminate eventually as corneal epithelial cells. Basal and wing cells replenish the superficial epithelial cells as they undergo apoptosis and slough into the tear film throughout normal desquamation. The former cells migrate in an apical direction from posterior and peripheral to anterior and central cornea (Eghrari et al., 2015). The β-4 integrin is differentially expressed on the membranes of LSCs and epithelial cells in the basal, basolateral and apical membranes of the cornea (Pajoohesh-Ganji et al., 2006; Stepp, 2006). As these cells differentiate to become wing and suprabasal cells, their β-4 integrin expression is downregulated (Stepp, 2006; Stepp et al., 2017). The epithelial cells expressing β-4 integrin are located in close proximity to subbasal axons, and it is hypothesized that these epithelial cells wrap around the axons in the corneal epithelium to act as surrogate Schwann cells (SCs) by providing axonal support (Stepp et al., 2017). The basal epithelial cells adhere to the underlying the epithelial basement membrane and stroma via hemidesmosomes (Torricelli et al., 2013; Eghrari et al., 2015).

Hemidesmosomes together with anchoring fibrils and filaments, form an anchoring complex with an extracellular matrix that is associated with the epithelial basement membrane and stroma (Torricelli et al., 2013). The epithelial basement membrane releases factors, transforming growth factor β-1 and platelet-derived growth factor (PDGF), that modulate epithelial cell differentiation and apoptosis. Primarily, the basement membrane functions to maintain adherence of the corneal epithelium to the underlying Bowman’s layer and corneal stroma via the anchoring complexes (Torricelli et al., 2013; Eghrari et al., 2015).
Three components are necessary to form the aforementioned hemidesmosome, and thus the anchoring complex (Figure 1-2). These are 1) an intracellular plaque protein 2) a transmembrane protein, and 3) basement membrane proteins that are associated with an extracellular matrix (Torricelli et al., 2013). The intracellular plaque proteins link elements of the intracellular cytoskeleton at the surface of the plasma membrane of the basal epithelial cells (Figure 1-2A). The transmembrane proteins, including α6β4 integrin and type XVII collagen, serve as cell receptors to connect the basal cell interior to the extracellular matrix (Figure 1-2B) (Stepp et al., 1990; Torricelli et al., 2013). These transmembrane proteins link the epithelial basement membrane with keratin intermediate filaments and laminin in the extracellular matrix (Figure 1-2C) (Stepp et al., 1990; Dowling et al., 1996; Stepp et al., 1996). Collagen VII is another major component of anchoring fibrils that extends into the extracellular matrix to attach to anchoring plaques located in the stroma (Figure 1-2D) (Gipson et al., 1987).
**Figure 1-2 Hemidesmosome anchoring complex.** In the cornea, this links the basal epithelial cells, epithelial basement membrane, and extracellular matrix and is composed of A. intracellular plaque proteins in the basal epithelial cells, B. transmembrane proteins, and C. basement membrane proteins connecting the basal epithelial cells, Bowman’s layer and underlying stroma D. Anchoring fibril components link the complex to anchoring plaques in the stroma. In this simplified diagram, not all molecules and proteins present in the corneal areas of the complex are included.

Expression of the proteins associated with the anchoring complex change during corneal epithelial wound healing (Fujikawa et al., 1984; Stepp, Zhu and Cranfill, 1996), and abnormalities of the corneal epithelial basement membrane are associated with recurrent epithelial breakdown (Payant et al., 1991; Colville et al., 1997; Resch et al., 2009). *In vitro* organ culture studies of rabbit corneas implicate corneal innervation in the maintenance of the expression of proteins required to form the anchoring complex (Nishida et al., 1996; Yamada et al., 2005).

**ii) Bowman’s Layer**

The Bowman's layer is acellular and non-regenerating (DelMonte and Kim, 2011; Eghrari et al., 2015). This 8-12 μm layer is composed of Type IV collagen, laminin, heparin sulfate and fibronectin collagen fibrils (Jacobsen et al., 1984). In humans, this layer is well developed compared to in rats and other species. However, there are no apparent differences in the aforementioned anchoring structure (Hayashi et al., 2002).

**iii) Stroma**

The stroma functions to provide structural support and maintain transparency in the cornea. In humans, the cornea is approximately 500 μm thick, the stroma comprising 80-90% of the corneal thickness (Maurice, 1970; Boote et al., 2003). In the stroma, parallel collagen fibers, fibrils, are arranged into parallel layers, or lamellae. The dense and precise arrangement of the collagen fibrils, organized into orthogonal lamellae (Maurice, 1957, 1970; Jester et al., 1999; Hassell and Birk, 2010), reduces the opportunity for light to scatter and thus promotes transparency. The arrangement of the lamellae varies with stromal depth and, because the posterior cornea is more hydrated than the anterior,
refraction decreases as light passes through the stroma. Anteriorly the lamellae are arranged into short, narrow sheets and extensively interconnected. Posteriorly, wide, thick lamellae extend from limbus to limbus without interlamellar connections (Eghrari et al., 2015).

There are 13 types of collagen fibrils in the stroma, the most common (58%) of which is Type I collagen (Komai and Ushiki, 1991; Meek and Fullwood, 2001; Robert et al., 2001; Fini and Stramer, 2005). Proteoglycans maintain the small distance between collagen fibrils. Proteoglycans function to help regulate hydration and promote corneal clarity, shape and volume (DelMonte and Kim, 2011).

Keratocytes reside between the lamellae, primarily in the anterior corneal stroma, and synthesize molecules necessary to maintain stromal integrity, collagen fibers and matrix metalloproteases (Jester et al., 1999; DelMonte and Kim, 2011). Keratocyte presence can contribute to light scattering in the stroma, which is reduced by corneal water-soluble crystallins (Jester et al., 1999; DelMonte and Kim, 2011; Eghrari et al., 2015). The crystallins are lost from keratocytes during stromal repair after injury (Stramer and Fini, 2004). Keratocytes are activated by stromal injury and behave like fibroblasts in regulating the healing response (Stramer and Fini, 2004). The keratocytes also synthesize matrix metalloproteases that are critical in stromal remodeling after injury because of their involvement in extracellular matrix remodeling, cell-matrix interaction, inflammatory cell recruitment and cytokine activation (Fini and Stramer, 2005; Gabison et al., 2005). The stroma also plays a role in immune regulation. Dendritic, and other bone marrow-derived cells, such as macrophages, reside in the corneal stroma (Hamrah et al., 2003b) and were shown to participate in immunity and inflammation in response to thermal cautery of the rodent cornea (Hamrah et al., 2003a; Hamrah and Dana, 2007).

Blood vessels are absent in the stroma, preserving stromal and corneal clarity. This avascularity is maintained by the balance of anti- and pro-angiogenic factors. For example, the pro-angiogenic vascular endothelial growth factor (VEGF) function is neutralized by its binding to corneal-secreted soluble VEGF receptor-1 (sVEGFR-1 or sflt-
1). Suppressing this receptor with antibodies or RNA interference in mice results in angiogenesis, and vascularization of the cornea, and thus impaired corneal clarity (Ambati et al., 2006).

iv) Descemet's membrane
The Descemet's membrane is a 10 μm layer that functions as the basement membrane for the corneal endothelium. The membrane is composed of two layers. The anterior banded layer is composed of Type IV and VIII collagen fibrils and proteoglycans. The posterior non-banded layer is laid down by endothelial cells. This membrane assists the endothelium in maintaining corneal dehydration (Eghrari et al., 2015).

v) Endothelium
The corneal endothelium comprises the deepest cellular layer of the cornea. It is a single cell layer of flat, polygonal cells that contribute to approximately 4-5 μm of the corneal thickness. The layer is essential in preserving corneal dehydration (DelMonte and Kim, 2011; Eghrari et al., 2015), which is important in the maintenance of corneal shape and thus clarity (Zucker, 1966). The endothelial cells are adherent to the overlying Descemets membrane via hemidesmosomes and to each other through lateral interdigitations, tight junctions and gap junctions (DelMonte and Kim, 2011; Eghrari et al., 2015). The gap junctions are involved in electrical coupling of endothelial cells (Polse et al., 1990) Na+/K+-ATPase and intracellular carbonic anhydrase pumps are also located on the lateral endothelial membranes and contribute to corneal dehydration (Stiemke et al., 1991). Corneal dehydration is a passive pump-leak process as fluid moves from the hypo-osmotic stroma and hypertonic aqueous humor. Although energy use is not directly necessary, maintenance of the hydration gradient is reliant on energy-requiring ion transport through the Na+/K+-ATPase and intracellular carbonic anhydrase pathways. These pathways create a net efflux of ions from the corneal stroma to the aqueous humor, facilitating fluid efflux from the cornea and thus corneal dehydration (Watsky et al., 1989; DelMonte and Kim, 2011).
Endothelial cell density declines with age (DelMonte and Kim, 2011; Eghrari et al., 2015). The surviving cells do not proliferate (Joyce, 2003) but their morphology adapts to occupy the space left by the degenerated cells. This change in morphology with age is associated with a reduced ability of the cornea endothelium to dehydrate the cornea (Polse et al., 1990; Gambato et al., 2014). In endothelial failure, there is corneal edema due to a net influx of ions and thus aqueous fluid into the cornea (DelMonte and Kim, 2011; Eghrari et al., 2015).

III - CORNEAL INNERVATION

The corneal epithelium is the most densely innervated tissue in the body. The corneal epithelial innervation is 300 to 600 times more dense than that of the skin epithelium, and 20-40 times than that in the tooth pulp (Rózsa and Beuerman, 1982; Guthoff et al., 2005; Stepp et al., 2017). The majority of corneal sensory innervation originates at the trigeminal ganglion (Figure 1-3). The innervating axons travel to the cornea through the ophthalmomaxillary nerve (V1) and, once in the orbit, they travel by way of the long ciliary nerves (Figure 1-3) (Müller et al., 2003), through the limbus to the cornea (Figure 1-4 A). A small proportion of corneal sensory innervation is sympathetic and parasympathetic, deriving from the superior cervical and ciliary ganglia, respectively (Figure 1-3). In both humans and rats, the sympathetic and parasympathetic fibers are localized to the peripheral cornea in small quantities (Toivanen et al., 1987; Marfurt et al., 1993; Marfurt et al., 1998).
Figure 1-3 Origin of corneal innervation. Corneal sensory innervation (green) originates at the trigeminal ganglion. The axons innervating the cornea travel to their destination via the ophthalmomaxillary nerve (V1) which enters and, in the orbit, via long ciliary nerves. The cornea receives some parasympathetic (blue) and sympathetic (orange) innervation from the ciliary and superior cervical ganglions (respectively).

Corneal sensory nerve fibers travel radially from the limbus, the limbic plexus (LP; Figure 1-4A), through the deep stroma to superficial epithelium, creating a centripetal pattern of corneal innervation (Figure 1-4, B) (Yang et al., 2018). The majority of innervation enters the cornea from the LP by way of the stroma (Figure 1-4 A). The innervating axons lose their myelination within 1 mm of entering the stroma, thereby promoting corneal clarity. Non-myelinating SCs in the stroma form a single layer of ensheathment around these axons (Müller et al., 1996; Müller et al., 2003). Within the stroma, the nerves branch extensively to form the sub-epithelial plexus (SEP) where the axons travel parallel to the surface of the cornea before they penetrate the Bowman’s layer and form the sub-basal plexus (SBP) between Bowman’s layer and the basal epithelium. Single intraepithelial axons then branch from the nerve fibers of the SBP to travel anteriorly through the corneal epithelium where they terminate as free nerve endings (FNE) (Figure 1-4).

The majority (~66%) of the sensory fibers innervating the cornea are unmyelinated C-fibers that terminate as polymodal nociceptors in the epithelium. These axons have large receptive fields attributed to their extensive axonal branching. The remaining are thinly myelinating, Aδ fibers that terminate as cold-thermal and mechano-nociceptors (Belmonte et al., 2004; Yang et al., 2018). The majority of the corneal nerve fibers terminate in the corneal epithelium. The intraepithelial nerve fibers travel perpendicular to the epithelial basement membrane (Figure 1-4 A,C) and terminate as free nerve endings in basal and suprabasal/wing epithelial cell layers (Stepp et al., 2017; Yang et al., 2018). A small population of nerve fibers in the cornea terminate in the stroma, some in close proximity to stromal keratocytes (Muller et al., 1996; Seyed-Razavi et al, 2014). In the peripheral nervous system, unmyelinated, unsheathed axons are termed free nerve endings (Stepp et al., 2017). In the skin, free nerve endings are typically shorter in length.
**Figure 1-4 Pattern of corneal innervation.** A. Sagittal-section of the cornea and limbus showing corneal innervation. Via the limbic plexus (LP), the corneal innervation enters the stroma to form the sub-epithelial plexus (SEP). From here axons penetrate the Bowman’s layer to form the sub-basal plexus (SBP) beneath the corneal epithelium. Single axonal fibers branch and terminate as free-nerve endings in the corneal epithelium. B (Muller *et al.*, 2003), C (Rozsa and Beurman, 1982) Diagrammatic representation of the distribution of the corneal innervation demonstrating the SEP that gives rise to the SBP, which branch into intraepithelial nerve terminals also known as FNEs in the B whole cornea, and C cellular level.
than 100 μm, and, in contrast they are as millimeters long in the corneal epithelium (Rózsa and Beuerman, 1982; Stepp et al., 2017). The plasma membrane of corneal epithelial basal cells wraps around individual axons and groups of axons (Figure 1-5) (Müller et al., 2003; Marfurt et al., 2010; Stepp et al., 2017) resembling the Remak bundles of non-myelinating SCs wrapping around axons in unmyelinated peripheral nerves (Shaheen et al., 2014; Stepp et al., 2017). These basal epithelial cells may act as surrogate SCs to provide axonal support to the intraepithelial axons (Stepp et al., 2017). Their terminals are dynamic, rearranging and re-growing in response to epithelial cell turnover and injury in mice (Harris and Purves, 1989; Pajoohesh-Ganjii et al., 2006; Yu and Rosenblatt, 2007; Namavari et al., 2011; Stepp et al., 2017).

Figure 1-5 Similarities between non-myelinating Schwann cells and corneal basal epithelial cells. Schematic representations of cell membranes of A non-myelinating Schwann cells and B corneal epithelial basal cells wrap around sensory axons. A. Cross-section of a non-myelinating Schwann cell, sometimes referred to as a Remak bundle, containing several axons. B. Cross-section of corneal epithelial basal cell membranes wrapping around single axons or clusters containing several axons. In these schematics, A and B are not shown at the same scale; axon (blue) diameters would normally be the same in both A non-myelinating Schwann cells and B corneal epithelial basal cells. (Reproduced from Stepp et al, 2017 with permission from Elsevier: License # 4595681499795)
Functional corneal innervation is critical for maintenance and repair of the corneal epithelium because corneal sensory nerves are an important source of trophic mediators (Figure 1-6). Previous studies have demonstrated a trophic influence of the trigeminal neurons on the corneal epithelium using co-cultures of corneal epithelial cells and dissociated trigeminal neurons (Chan and Haschke, 1981, 1982; Ko et al., 2013; Kowtharapu et al., 2014). The following nerve-derived peptides are suggested to play a role in corneal epithelial maintenance and repair (Müller et al., 2003):

**Figure 1-6 Key players in corneal epithelial maintenance and repair.** Sagittal-section of the cornea and limbus. Substance P (SP) and calcitonin gene-related peptide (CGRP) are co-localized in corneal sensory nerves and promote epithelial cell (purple/pink) proliferation and migration. Schwann cells (red) ensheath the axons (yellow) in the limbus and corneal layers deeper than the epithelium. Nerve growth factor (NGF) is a neurotrophic factor that is thought to promote epithelial differentiation and proliferation in the cornea, but its source is unknown. NGF’s trkA receptor is found in high concentrations in the basal epithelium of the limbus where the limbal stem cells (light blue) are found.
Substance P (SP) is a peptide which is present, at physiologically relevant levels, in the normal healthy cornea (Figure 1-6) (Tervo et al., 1981; Elbadri et al., 1991; Marfurt and Echtenkamp, 1995; Marfurt et al., 2001; Müller et al., 2003). Corneal epithelial cells express receptor NK-1 which mediates SP function (Figure 1-6) (Nakamura, Ofuji, et al., 1997; Mantyh, 2002; Yang et al., 2014). In culture, SP stimulates epithelial cell proliferation (Reid et al., 1993; Garcia-Hirschfeld et al., 1994).

In vitro SP upregulates the expression of proteins that are necessary to maintain tight-junctions in the corneal epithelium (Araki-Sasaki et al., 2000; Ko et al., 2009). The peptide is implicated in the formation corneal epithelial tight junctions, by upregulating the expression of ZO-1 (Ko et al., 2009). In addition, SP, in conjunction with insulin-like growth factor-1 (IGF-1), increases the expression of α5 integrins and E-cadherin, which is required for epithelial adhesion to fibronectin in the extracellular matrix (Nakamura et al., 1998; Chikama et al., 1999; Araki-Sasaki et al., 2000). Administration of SP in vitro upregulates integrin expression on the basal corneal epithelial cells and increases the attachment of these cells to proteins of the epithelial-stromal anchoring complex (Nishida et al., 1992, 1996; Yamada et al., 2005).

During corneal epithelial wound healing, SP in conjunction with IGF-1 stimulates epithelial cell attachment to anchoring complex proteins of the newly wounded area (Juhasz et al., 1993; Nakamura et al., 1998), thus aiding in epithelial cell migration. The synergistic facilitation of epithelial cell migration by SP and IGF-1 has been confirmed both in vitro and in vivo experiments (Nakamura et al., 1998; Chikama et al., 1999). SP, SP analogues, and IGF-1 have all been used to treat persistent epithelial defects (PEDs) in human patients (Brown et al., 1997; Kingsley and Marfurt, 1997; Chikama et al., 1998; Morishige et al., 1999; Murphy et al., 2001; Yamada et al., 2008). It is therefore apparent that nerve derived SP plays a role in maintaining epithelial integrity.

Calcitonin gene-related peptide (CGRP) is often co-localized with SP in corneal sensory nerves (Figure 1-6) (Beckers et al., 1993; Marfurt, Murphy and Florczak, 2001; Müller et al., 2003), and its receptors are abundant in the corneal and limbal epithelium
The concentration of CGRP in human tears increases following corneal epithelial injury (Mertaniem et al., 1995). After injury, exogenous CGRP application has been shown to increase corneal epithelial cell DNA synthesis and migration in some animal studies (Reid et al., 1993; Mikulec and Tanelian, 1996), but not in others. However, the studies that found no effect of the peptide on cell migration, did not co-administer CGRP with other trophic peptides, such as SP (Nishida et al., 1996). This suggests that CGRP may only work synergistically with SP or other trophic factors to increase corneal epithelial cell migration. CGRP and SP may also have an inflammatory role following corneal injury by stimulating IL-8 secretion (Tran et al., 2000; Tran et al., 2000). Although CGRP alone may or may not elicit an effect on the corneal epithelium, it could possibly potentiate the function of other trophic factors.

Thickness of the corneal epithelium (Alper, 1975), and proliferation of corneal epithelial and LSC after injury (Park et al., 2006; Yin et al., 2011; Ueno et al., 2012) are influenced by corneal innervation. Corneal wound healing is significantly impaired in animal models of corneal hypoesthesia, including diabetes mellitus 1 and neurotrophic keratopathy (NK) (Mikulec and Tanelian, 1996; Nagano et al., 2003; Nakamura et al., 2003). Nerve-derived peptides, including SP and CGRP, have demonstrated trophic influences on the corneal epithelium during normal epithelial cell turnover and are increased in concentration following injury of the normally innervated cornea (Mertaniem et al., 1995). SP promotes corneal wound healing in rats with type I diabetes mellitus by activating the epidermal growth factor (EGF) signaling pathway (Yang et al., 2014) that is implicated in the turnover of corneal epithelial cells (Nakamura, Nishida, et al., 1997; Peterson et al., 2014; Rush et al., 2014) and wound healing (Zieske et al., 2000). In support of these findings, a study demonstrated that the receptor for EGF was not activated in corneas of type I diabetic rats and, in turn, which could account for the impaired healing of the debrided corneal epithelium (Xu nd Yu, 2011). Re-activation of this pathway with SP promotes corneal wound healing in rats with type I diabetes mellitus (Yang et al., 2014), providing further evidence for a role of the EGF signaling pathway in the normal turnover and healing of the corneal epithelium.
Other neuropeptides and neurotransmitters that have been studied in the cornea include norepinephrine, acetylcholine and vasoactive intestinal polypeptide. Ample evidence suggests these factors as well as SP and CGRP play a critical role in the maintenance and repair of the corneal epithelium (Figure 1-6) (Müller et al., 2003) In the cornea, several neurotrophins, including nerve growth factor (NGF) (Figure 1-6), neurotrophin 3 and 4/5 (NT-3 and NT-4), and brain derived trophic factor, and their receptors have been confirmed at the transcription and protein levels (Lambiase et al., 1998). NGF in particular is of interest due to its effectiveness as a topical agent in treating persistent epithelial defects (PEDs) in animal models (Lambiase et al., 2000; Esquenazi et al., 2005) and in patients (Lambiase et al., 1998; Bonini et al., 2000; Tan et al., 2006; Lambiase, Sacchetti and Bonini, 2012; Sacchetti et al., 2017). NGF stimulates epithelial cell proliferation and differentiation in vitro (F. E. Kruse and Tseng, 1993; You, Kruse and Völcker, 2000), and although the source of NGF in the cornea unknown, several have been suggested in the literature, including corneal epithelial cells, corneal nerves (Figure 1-6) and tears (Müller et al., 2003). The SCs may also be a source (Figure 1-6), although this has yet to be established.

V - LIMBAL STEM CELLS

Corneal epithelial homeostasis is dependent on the distinct population of stem cells that are located within a stem cell niche in the Palisades of Vogt of the limbus (Fig. 1-7 A-i) (Dua et al., 2005; Ahmad, 2012). Corneal epithelial wound healing is significantly impaired in the absence of the limbal epithelium (Huang and Tseng, 1991). Limbal epithelial stem cells (LSCs) increase and then decrease one and four days after injury, respectively Cotsarelis et al., 1989; Lehrer e al., 1998; Park et al., 2006). Corneal epithelial cells also increase and decrease but a day later than the LSCs (Park et al., 2006). The corneal epithelial cells in turn, migrate centripetally to replenish the corneal epithelium in response to both desquamation and corneal injury (Thoft and Friend, 1983; Collinson et al., 2004; Di Girolamo et al., 2015). The XYZ Hypothesis proposes that, in order to maintain corneal epithelial homeostasis, cell loss (Z) must be balanced by cell replacement through stem cell proliferation and differentiation (X) and migration to the central cornea (Y) (Thoft and
In animal models, LSCs are often characterized as slow-cycling, retaining BrdU, and they demonstrate the morphology of a typical stem cell (Romano et al., 2003; Chen et al., 2004; Schlötzer-Schrehardt and Kruse, 2005; Bentley et al., 2007). These LSCs differentiate into transient amplifying cells (TACs) that are fast-dividing cells in the limbal basal epithelium and in the peripheral cornea (Fig. 1-7A). TACs are further differentiated into post-mitotic and terminally differentiated corneal epithelial cells in the more central cornea (Fig. 1-7A) (Schlötzer-Schrehardt and Kruse, 2005).

Figure 1-7 Limbal stem cell function. A. (i) Limbal stem cells (LSCs; light blue), located in their niches in the basal limbal epithelium, (ii) proliferate and differentiate into transient amplifying cells (navy blue) that migrate towards the central cornea in the basal epithelium, above the Bowman’s Layer (green). (iii) These cells further differentiate into post-mitotic (purple) and, finally, terminally differentiated corneal epithelial cells (pink). B. Diagram of LSC markers as LSCs transition from resting LSCs to transient amplifying cells prior to becoming terminally differentiated into corneal epithelial cells, based on published literature (Di Iorio et al., 2005; Barbaro et al., 2007; Menzel-Severing et al., 2018)
Stem cells in various tissues, including bone marrow can be identified by their ability to take up Hoechst 33342 dye (Goodell et al., 1996). Likewise, the LSCs have likewise been identified with this dye (Ueno et al., 2012). Whilst there are no definitive LSC markers, ABCB5 (Ksander et al., 2014; Frank and Frank, 2015), ABCG2, Hes1, p63, p75NTR and trkA have been accepted as distinguishing markers for the LSCs in vivo (Schlötzer-Schrehardt and Kruse, 2005; Takács et al., 2009). More recently, groups have identified other markers, such as C/EBPδ, Bmi1, SOX9, ΔNp63α that have been shown to identify resting LSCs and ΔNp63α that also identifies activated LSCs/early TAC cells, and finally, ΔNp63β, ΔNp63γ and Wnt/β-catenin that identifies TACs (Di Iorio et al., 2005; Barbaro et al., 2007; Menzel-Severing et al., 2018). Thereby, the differential expression of LSC markers is able to identify and follow the state of activation and differentiation of LSCs (Figure 1-7B).

The activation of LSCs promotes corneal epithelial wound closure (Mort et al., 2012; Ljubimov and Saghizadeh, 2015). Whilst their numbers have been suggested to decrease in denervated corneas of mice (Ueno et al., 2012), patients with limbal stem cell deficiency (Dua et al., 2000; Menzel-Severing et al., 2018) and rabbits with surgically removed limbal epithelium in innervated cornea (Huang and Tseng, 1991) exhibit corneal vascularization and conjunctivalization which are not typical symptoms of NK (Figure 1-8). These symptoms are also elicited in the normally innervated cornea by damaging the LSC niche with localized alcohol application in mice but, removing LSCs surgically in the innervated cornea without damaging the niche, resulted in dedifferentiation of the corneal epithelial cells and repopulation of the LSC niche (Nasser et al., 2018). This study indicates that corneal epithelial homeostasis is dynamic, relying on functioning corneal epithelial cells, LSCs and the LSC niche.
In contrast to the sensory nerve innervation of corneal epithelial cells being essential for maintaining their function, the role in limbal function is not understood. Some studies indicate that innervation is important for limbal function. The sensory nerve endings in the limbus are compact nerve endings that are located in close proximity to the Palisades of Vogt (Al-Aqaba et al., 2018). The authors noted that these endings are typical of rapidly-adapting low-threshold mechanoreceptors which are encapsulated by differentiated Schwann and/or endoneurial/perineurial-related cells. The LSCs may be directly correlated with this innervation because the severity of the symptoms of LSC deficiency in patients correlated with reduced corneal sub-basal nerve density and increased nerve tortuosity (Chuephanich et al., 2017).

The evidence that 1) LSCs function to maintain corneal epithelium, 2) the disease symptoms of LSC deficiency and NK differ, and 3) the ill-defined relationship between LSCs and sensory nerve innervation, suggests that NK patients are not LSC deficient or that the LSC niche is damaged. Rather, LSC function may be impaired.

VI - SCHWANN CELLS IN THE CORNEA

Both myelinating and unmyelinating Schwann cells (SCs) are present in the limbus. Within 1 mm of entering the corneal stroma, the axons lose their myelination. In the cornea, unmyelinating SCs continue to ensheath the axons in the sub-epithelial and subbasal plexus (Müller et al., 2003), but are not present in the corneal epithelium. It is
suggested the basal epithelial cells act as surrogate SCs, by responding to epithelial and intraepithelial axonal injury via mechanisms similar to those induced by SCs in response to axonal injury and during Wallerian Degeneration (Stepp et al., 2017). Although the presence of SCs is noted, little is known about their role in the limbus and/or cornea, and the SC response to corneal denervation remains to be determined.

SCs have been extensively studied in other tissues (Jessen et al., 2015; Jessen and Mirsky, 2016; Carr and Johnston, 2017). These cells provide axonal support in the form of myelination or the formation of Remak (non-myelinating) bundles (Jessen, Mirsky and Lloyd, 2015; Harty and Monk, 2017). SCs contribute to the repair process after nerve injury by releasing neurotrophic factors such as NGF, brain-derived neurotrophic factor, NT-3, VEGF and others (Meier et al., 1999; Fontana et al., 2012; Brushart et al., 2013). It is suggested that axonal injury stimulates the de-differentiation of SCs (Stoll and Müller, 1999). De-differentiated SCs have been shown to play a critical role in tissue regeneration in the rodent digit tip (Johnston et al., 2016) and to promote healing of an epithelial injury in skin (Johnston et al., 2013). SCs have been found to support hematopoietic stem cells in the bone marrow (Yamazaki et al., 2011; Yamazaki and Nakauchi, 2014), and a relationship between SCs and stem cells in other tissues has been hypothesized due to their anatomical proximity (Carr and Johnston, 2017).

SCs have a critical function in other tissues and injury models, and their physical presence in the cornea and limbus suggests they may play an important role in maintaining limbal and corneal health. It’s important to note that if corneal epithelial basal cells act as surrogate SCs, as has been suggested by Stepp et al. (2017), that they too may play an important role in maintaining corneal health as we proposed above.
1.4 Neurotrophic Keratopathy

I. INTRODUCTION

In this subchapter, neurotrophic keratopathy (NK), the corneal disease investigated in this thesis, is discussed in detail.

II. ETIOLOGY, DIAGNOSIS, PROGNOSIS

In the absence of corneal sensory innervation, patients lack protective reflexes and the nerve-derived trophic mediators that support corneal epithelial maintenance and healing. Consequently, these patients develop NK that is characterized by corneal anesthesia or hypoesthesia, recurrent or persistent epithelial defects (PEDs), progressive stromal scarring and can eventually lead to blindness (Bonini et al., 2003; Ramaesh et al., 2007; Sacchetti and Lambiase, 2014) (Figure 1-9). NK can be congenital or develop as a result of corneal nerve injury. Corneal nerve injury can be a result of viral infection, chemical burns, intracranial trigeminal nerve injury or corneal surgery. Systemic diseases that affect the peripheral nervous system, such as diabetes, multiple sclerosis or leprosy, can also cause axonal degeneration in the cornea, resulting in NK (Table 1-1).

![Figure 1-9 Symptoms of neurotrophic keratopathy.](image_url) In neurotrophic keratopathy, patients exhibit A. corneal epithelial ulcerations and breakdown, which can lead to B. progressive scarring and vision loss. Photographs from the Hospital for Sick Children.
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<th>Causes/etiology of neurotrophic keratopathy</th>
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<td>- Laser-assisted in situ keratomileusis (LASIK)</td>
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<td>- Corneal incision</td>
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Table 1-1 Causes/etiology of neurotrophic keratopathy

The prevalence of NK is five in 10,000 people worldwide (Sacchetti and Lambiase, 2014). Because patients have absent corneal sensation, they often fail to present for care. As a result, several patients remain undiagnosed until scarring of the cornea results in visual decline. For a clinical diagnosis of NK, patients must present with both corneal epithelial ulcerations and absent or significantly impaired corneal sensation. Corneal sensitivity is traditionally measured with Cochet-Bonnet esthesiometry by recording the patient response to pressure on the ocular surface using a nylon filament of variable length (0-6 cm) (Norn, 1975; Sacchetti and Lambiase, 2014; Semeraro et al., 2014; Mastropasqua et al., 2017, 2018; Versura et al., 2018). First, sensitivity is measured at the
esthesiometer’s full length of 6 cm, and its length is decreased in steps until the patient reports sensation or contact. This is repeated in the central cornea as well as the four quadrants of the cornea: superior, temporal, inferior, nasal (Figure 1-10). Normal corneal sensation is considered as between 5-6cm, whereas patients with corneal anesthesia or hypoesthesia tend to report sensation between 0-3cm (Dua et al., 2018). More recently, CO\textsubscript{2} gas esthesiometry has been developed to record patient response to a burst of CO\textsubscript{2} gas. The latter esthesiometer enables a non-contact evaluation of mechanical, chemical and thermal neuroreceptors in the cornea by varying pressure, CO\textsubscript{2} concentration and temperature, respectively (Tesón et al., 2012). In vivo confocal microscopy can also be used to evaluate and document the absence of corneal sensory innervation in the affected cornea(s) of NK patients (Villani et al., 2014). Corneal epithelial ulcerations can be detected with topical fluorescent dye, which stains the underlying basement membrane or stroma, and slit lamp microscopy (Sacchetti and Lambiase, 2014).

Clinically, according to the Mackie (1995) classification, NK is classified into three stages based on the severity of the disease. Stage I is characterized by superficial punctate keratopathy that over time can progress to stromal scarring, and stage II by PEDs. Stage III involves corneal ulcerations that progress to extensive stromal injury, including perforation and stromal melting (Mackie 1995). The cause and severity of the disease determine the prognosis of, and treatment for, NK patients.

III - MOLECULAR BASIS OF DISEASE
Corneal sensory nerves are an important source of trophic mediators as described above in Chapter 1.3.IV (Figure 1-6). Studies have shown that the absence of corneal innervation, in animal models, results in the thinning and breakdown of the corneal epithelium, and impairs corneal wound healing (Sigelman and Friedenwald, 1954; Alper, 1975). Within 24 hours of trigeminal ganglion ablation in a mouse model of NK, degeneration of the corneal axons is apparent, and the corneal surface starts to become cloudy (Ferrari et al., 2011). In the absence of corneal sensory innervation, such as in NK, corneal epithelial maintenance and healing after injury are impaired (Sigelman and Friedenwald, 1954; Alper, 1975). This impairment may arise from a depletion of factors such as those listed below (Figure 1-6):

**Substance P (SP)**, described in Chapter 1.3.IV, is decreased in tears of patients with impaired corneal sensation (Yamada et al., 2000). Subcutaneous injections of capsaicin, which acts to deplete SP from axon terminals, has, in previous studies with mice, resulted in corneal changes consistent with corneal hypoesthesia and NK (Fujita et al., 1984; Shimizu et al., 1987). SP co-localizes with sensory axons in the normal, healthy cornea (Tervo et al., 1981, 1982; Lehtosalo, 1984), and functions to stimulate epithelial cell proliferation (Reid et al., 1993; Garcia-Hirschfeld, Lopez-Briones and Belmonte, 1994), upregulate epithelial cell migration, in conjunction with insulin-growth factor 1 (IGF-1) (Nakamura, Nishida, et al., 1997; Chikama, Nakamura and Nishida, 1999) and increase the expression of tight-junction proteins in the corneal epithelium (Araki-Sasaki et al., 2000; Ko, Yanai and Nishida, 2009). Thus, the corneal changes consistent with NK in the depletion or blocking of SP (Fujita et al., 1984; Shimizu et al., 1987), is presumably due to the impaired epithelial cell proliferation, migration, and adhesion.

**Calcitonin gene-related peptide (CGRP)** co-localizes with SP in corneal sensory nerves (Beckers et al., 1993; Marfurt, Murphy and Florczak, 2001; Müller et al., 2003; He and Bazan, 2016) and, as described in Chapter 1.3, acts synergistically with other neuropeptides and neurotrophins to facilitate epithelial cell migration (Nishida et al., 1996;
Nakamura et al., 1997; Tran et al., 2000; Tran et al., 2000). CGRP depletion in absent corneal innervation can thus contribute to NK symptoms.

**Nerve Growth Factor (NGF)** is a neuropeptide suggested to be released from a variety of possible sources: corneal sensory neurons (Lambiase et al., 1998), the corneal epithelium (Lambiase et al., 2000), corneal tears (Vesaluoma et al., 2000), and corneal SCs (Chapter 1.3.VI). NGF’s TrkA receptors are localized in the basal epithelium of the limbus (Touhami et al., 2002; Qi et al., 2008). In the limbal epithelium exists the Palisades of Vogt, the LSC niche (Ahmad, 2012), suggesting that NGF may act on these LSCs to exert its healing effect on the cornea. NGF promotes corneal wound healing by stimulating epithelial proliferation and differentiation (Blanco-Mezquita et al., 2013). In NK, there is an impairment in epithelial healing following corneal injury, thus implicating innervation in the NGF-mediated LSC-dependent epithelial healing (Sacchetti et al., 2017).

**IV - ANIMAL MODELS OF NEUROTROPHIC KERATOPATHY**

There are several animal models of NK that involve complete or impaired corneal denervation. The precise anatomy of corneal innervation was established in rabbit and macaque monkey by direct observation (Zander and Weddell, 1951a) after which the response to nerve injury was studied to establish the origin of the sensory innervation (Zander & Weddell, 1951b). Several models of injury were used: corneal autografting, corneal section (keratotomy), section or avulsion of the infraorbital nerve, ciliary nerve transection, superior cervical sympathetic ganglion extirpation, and Gasserian (trigeminal) ganglion destruction by partial removal of the temporal lobe of the cortex. Destruction of the trigeminal ganglion or ciliary nerve transection, but not infraorbital nerve transection or superior cervical ganglion extirpation, was shown to result in complete corneal denervation. Nerve transection caused such severe NK symptoms that all of the animals had to be sacrificed. The ganglionic destruction was also associated with high mortality, but with a protective eyelid suture, tarsorrhaphy, in the surviving animals, complete absence of sensation was reported after three weeks with a small number of
regenerating fibers in the cornea (Zander and Weddell, 1951b). Hence, both injury models are not appropriate NK animal models. Intracranial transection of the trigeminal ganglion in monkeys as compared to transection at lower levels of the corneal innervation was shown to be successful in immediate denervation of the cornea (Alper, 1975). Either intra- or extra-dural approaches in rats resulted in their mortality 24 hours later due to intracranial bleeding from vessels located near the trigeminal ganglion (Sigelman and Friedenwald, 1954). In contrast, a transpalatal intra-oral approach to cauterize and thereby injure the ophthalmic branch (V1) of the trigeminal ganglion was successful in denervating 60% of the corneas. Success with transpalatal thermo-coagulation of the medial trigeminal ganglion was reported in rats (Keen et al., 1982) and rabbits (Beuerman and Schimmelpfennig, 1980; Schimmelpfennig and Beuerman, 1982). Circumferential transection of all nerves to the rabbit cornea via limbus incisions while being less invasive, resulted in partial, and not complete, corneal denervation and in subsequent reinnervation (Chan-Ling et al., 1987). Immediate success with all these various techniques with the exception of the radial nerve transection was not followed by long-term assessments of corneal nerve regeneration.

Systemic and subcutaneous capsaicin injection in neonatal animals results in impaired corneal sensation, ulceration, neovascularization, and opacification and, hence, has also been used as a model of NK (Keen et al., 1982; Fujita et al., 1984; Gallar et al., 1990; Ogilvy et al., 1991; Marfurt et al., 1993; Donnerer et al., 1996; Nakamura et al., 2003; Lambiase et al., 2012). Nociceptive nerve endings, such as the A-δ and C unmyelinated sensory fibers that terminate in the corneal epithelium (Zander and Weddell, 1951a; Tervo and Tervo, 1981; Rózsa and Beuerman, 1982), are stimulated by capsaicin via their binding to transient receptor potential vanillin type 1 (TRPV1) (Hiura et al., 2002). Capsaicin binding to TRPV1 in neonatal rats induces retrograde neuronal death of sensory neurons in the trigeminal ganglion (Hiura et al., 2002). Topical capsaicin administration has also been used in adult models of corneal hypoesthesia (Gallar et al., 1990). Capsaicin treatment resulted in SP depletion and desensitization of corneal C-polymodal nociceptors and some Aδ nociceptors (Belmonte and Giraldez, 1981; Donnerer et al., 1996), but not of non-specific acetylcholinesterase-positive nerves.
Therefore, the latter nerves are believed to have functional properties other than chemical nociception (Hiura and Nakagawa, 2004, 2012; Nakagawa et al., 2009).

Although less invasive, capsaicin administration as a method of corneal denervation has several disadvantages. Gallar et al., (1990) demonstrated corneal epithelial wound healing was impaired by combined retrobulbar and topical capsaicin administration, but neither method of administration impaired healing alone. No study has investigated the density of corneal innervation following topical capsaicin application but, neonatal capsaicin administration in rats resulted in immediate corneal denervation (Ogilvy et al., 1991; Marfurt et al., 1993; Hiura and Nakagawa, 2005). However, several weeks following the neonatal treatment the affected cornea was reinnervated despite persistent keratitis at the same time point (Ogilvy et al., 1991; Marfurt et al., 1993; Hiura and Nakagawa, 2004, 2005). This re-innervation was attributed to sprouting of the remaining axons after neonatal capsaicin administration (Marfurt et al., 1993; Hiura and Nakagawa, 2005). Neonatal capsaicin application was less successful than V1 transection in achieving corneal denervation (Keen et al., 1982).

Ablation of corneal innervation using a stereotactic frame is a promising technique because there is less chance for human error in achieving corneal denervation. Transcranial (Tervo et al., 1979; Nagano et al., 2003) and transpalatal (Wong et al., 2004) stereotactic ablation of V1 via thermo-coagulation has been performed successfully in rats. Corneal denervation was confirmed with absent blink reflexes and clinical signs of NK (Tervo et al., 1979; Nagano et al., 2003; Wong et al., 2004). Tervo et al., (1979) observed complete corneal denervation up to eight days post-operatively. More recently, Ferrari et al (2011) described a model of transcranial ablation of V1 in mice. In this model, electrocautery was used to successfully denervate the cornea as evaluated by absence of blink reflex, decreased epithelial proliferation and documented corneal denervation. However, the study did not quantitatively assess nerve density before and after V1 nerve ablation (Ferrari et al., 2011). Transcranial and transpalatal corneal denervation procedures, although successful, do not allow for visualization of the base of the trigeminal ganglion and ophthalmic branch to confirm V1 injury.
To optimize the visualization of injury to corneal innervation, Yamaguchi et al. (2013) describe exposing the posterior orbit and transecting the long ciliary nerves. Transection of these long ciliary nerves resulted in significant corneal denervation. However, the absence of innervation has only been documented only up to two weeks after transection (Yamaguchi et al., 2013).

Our laboratory developed a novel rat model of NK (Figure 1-11) in which complete corneal denervation is observed four weeks after the initial procedure. In this rat model, denervation is achieved through stereotactic electrocautery of V1. The stereotactic procedure is repeated three weeks after initial ablation to prevent spontaneous regeneration of native sensory nerves. Stromal and sub-basal nerve fibers were quantified and were significantly decreased as compared to normally innervated corneas. The four week denervation period is the longest documented in an animal model, and allows for evaluation and comparison between our rat model of NK with and without corneal neurotization treatment (J. Catapano et al., 2018), as described in Chapter 1.5.

**Figure 1-11 Model of neurotrophic keratopathy (NK).** Corneal denervation in our model of NK is achieved by stereotactic ablation of the ophthalmomaxillary nerve (V1), originating from the trigeminal ganglion (TG). A. Stereotactic set-up used for corneal ablation B. Intracranial ablation of V1.
1.5 Treatments for Neurotrophic Keratopathy and Corneal Neurotization

I – INTRODUCTION

Conventional NK treatment focuses on preventing the progression of corneal damage but fails to provide a long-term solution to the disease. Corneal neurotization in NK patients is a novel surgical procedure that treats the underlying cause of NK by restoring corneal innervation, and thus sensation, to the NK cornea.

II - MANAGEMENT AND TREATMENT OF NEUROTROPHIC KERATOPATHY

Early diagnosis is critical for NK patients, yet due to the absence of sensation, thus pain, patients often fail to present for care until the disease progresses to corneal scarring and vision loss (Reviewed by Ramaesh et al., 2007; Davis and Dohlman, 2014; Semeraro et al., 2014; Versura et al., 2018). Initial treatment aims to prevent the corneal epithelium from injury and desquamation and to promote healing with the use of lubricants and pharmacological agents. The first line of defense for Stage I NK involve preservative-free artificial tears (Reviewed by Davis and Dohlman, 2014; Semeraro et al., 2014).

Once NK progresses to Stage II, and PEDs are present, treatment is focussed on epithelial healing and prevention of worsening ulcerations. In the presence of existing punctate keratopathy, a scleral (Grey et al., 2012) or corneal lens, or protective glasses can be used to protect the corneal surface (Donnenfeld et al., 1995; Ramaesh et al., 2007). Because inflammation has been shown to diminish epithelial healing in the absence of sensory innervation (Cavanagh et al., 1979), anti-inflammatory treatments, such as topical corticosteroids have been suggested in treating NK (Ramaesh et al., 2007; Sacchetti and Lambiase, 2014). Several studies suggest an improvement in the rate of epithelial wound healing in response to topical steroid treatments in both animals and humans (Ashton and Cook, 1951; Gasset et al., 1969; Phillips et al., 1983). However, this improvement is dependent on the dose of treatment (Ashton and Cook, 1951; Gasset
et al., 1969; Sugar et al., 1985). Steroids have also been shown to inhibit stromal healing and endothelial recovery and thus, may increase risk of stromal melting or perforation (Ashton and Cook, 1951; Gasset et al., 1969). Non-steroidal anti-inflammatory agents, in contrast, neither impair nor improve corneal wound healing (Srinivasan, 1982; Hersh et al., 1990). To avoid the toxic effect of topical steroid treatments, lubricants such as preservative-free artificial tears are continued in conjunction with prophylactic antibiotics (Semeraro et al., 2014; Mastropasqua et al., 2017).

Once NK progresses to stage III, surgical interventions are used to preserve ocular integrity for persistent and recurrent corneal ulcerations. To prevent epithelial ulceration from desiccation and desquamation, partial or complete tarsorrhaphy, surgical closure of the eyelids, is performed. Eyelid injection with botulinum toxin can provide a protective ptosis, namely the falling of the eyelid, to cover the insensate cornea (Kirkness et al., 1988). In cases involving stromal injury or corneal perforation, conjunctival flaps are raised to cover the corneal abrasion and sutured in place (Gundersen and Pearlson, 1969). Surgical amniotic membrane transplants on the corneal surface promote epithelial healing in cases of refractory corneal ulcers (Lee and Tseng, 1997; Khokhar et al., 2005). Perforations of less than 3 mm are closed with cyanoacrylate glue (Fogle et al., 1980; Ramaesh et al., 2007; Sacchetti and Lambiase, 2014; Semeraro et al., 2014). Corneal transplantation may be required in patients with more severe stage III NK to restore corneal architecture. However, the success of these corneal grafts is poor in this NK patient population. This is due to the nature of the disease, namely the absence of corneal innervation and thus the trophic influence of corneal sensory nerves, that often results in recurrent ulceration and scarring of the corneal graft (Jhanji et al., 2011; Lambley et al., 2014).

Recent interventions have been targeted at utilizing biological agents to restore the trophic mediators normally provided by corneal sensory innervation. Topical SP, in conjunction with IGF-1, has been used to treat corneal ulcers in patients presenting with PEDs (Brown et al., 1997; Chikama et al., 1998; Yamada et al., 2008), and SP was shown to work synergistically with IGF-1 to heal corneal ulcers in animal models of spontaneous
chronic corneal epithelial defects in dogs (Murphy et al., 2001), NK achieved by corneal

denervation in rats (Nagano et al., 2003) and capsaicin-induced NK in rats (Nakamura et al., 2003). Growth factors, such as NGF, increase corneal and limbal epithelial cell

proliferation \textit{in vitro} (Kruse and Tseng, 1993), and promote epithelial healing after corneal

injury in rats (Lambiase et al., 2000). Topical application of exogenous NGF has been

used to treat corneal epithelial ulcers in patients with NK. NGF eye drops are well

tolerated in patients (Ferrari et al., 2014) and, in response to NGF treatment, patients

suffering from NK and other ocular surface diseases demonstrated improved corneal

sensitivity and reduced corneal ulceration in clinical trials (Lambiase et al., 1998; Bonini

et al., 2000; Tan et al., 2006; Lambiase et al., 2012; Sacchetti et al., 2017). However, this

topical NGF treatment primarily targets patients and animal models that present with

corneal hypoesthesia. In a case of congenital NK, topical application of exogenous NGF

significantly improved epithelial healing but, this treatment alone was insufficient to

prevent progressive stromal opacification (Tan et al., 2006). Autologous serum contains

nerve-derived trophic factors and growth factors such as SP, IGF-1 and NGF (Matsumoto

et al., 2004). It is used clinically to treat NK patients and seems to restore epithelial

integrity and improves healing of PEDs, further implicating improved treatment efficacy of

a combination of these factors (Tsubota et al., 1999; Matsumoto et al., 2004; Soni and

Jeng, 2016). Platelet rich plasma and umbilical cord serum have similarly been shown to

improve corneal epithelial health, perhaps more effectively than autologous serum (Soni

and Jeng, 2016; Giannaccare et al., 2017).

The aforementioned treatment options fail to address the underlying cause of NK (lack of

innervation) and require daily ophthalmic management and frequent physician follow-up.

Despite optimal ophthalmic management, patients continue to develop ulcerations which

can lead to progressive scarring and eventually vision loss.

\textbf{III - CORNEAL NEUROTIZATION AND OTHER METHODS OF RE-INNERRVATION}

The first known attempt at surgical re-innervation of the cornea was reconstruction of the

damaged ophthalmic nerve (Samii, 1981). The procedure that was used involves a
craniotomy to expose the damaged intracranial ophthalmic nerve and subsequently transecting and coapting a sural nerve graft to the extracranial occipital nerve. The authors reported improved vision and corneal health, but no improved corneal sensation post-operatively. Further, the injured ophthalmic nerve is difficult to access, and the operative approach causes significant patient morbidity.

Neurotization is a surgical procedure in which a healthy donor nerve is used to restore motor and/or sensory function in a tissue lacking innervation. This procedure is successful for a range of clinical conditions including brachial plexus injuries (Wahegaonkar et al., 2007), facial paralysis (Hontanilla, Marre, and Cabello 2014) and more. Terzis et al (2009) described neurotization as a means of treating NK. This procedure involves reinnervating the NK cornea with the patients’ contralateral supratrochlear and supraorbital nerves. The procedure involves a bicoronal incision which is necessary to dissect enough length from the donor nerves to re-innervate the contralateral cornea. The donor nerves are tunnelled to the contralateral eye, separated into fascicles, and sutured to the conjunctival sac adjacent to the corneal limbus. Six patients suffering from NK for a mean (± standard deviation) of 7 ± 8.56 years were treated and demonstrated improvement in corneal sensation, visual acuity and corneal health post-operatively at 2.80 +/- 2.17 years post-operatively (Terzis et al., 2009). In one recent case, Gennaro et al., 2019 adapted Terzis' direct technique to use the homolateral infraorbital nerve to directly reinnervate the cornea in combination with facial reanimation procedure (Gennaro et al., 2019). Few other reports have published using Terzis' technique to treat NK (Allevi et al., 2014; Jacinto et al., 2016). This may be due to the disfiguration and invasiveness associated with the bicoronal incision required in this technique.

To eliminate the need for a bicoronal incision, Terzis’ direct corneal neurotization technique was modified by Borschel, Zuker, and Ali at The Hospital for Sick Children (Figure 1-12) (Elbaz et al., 2014; Bains et al., 2015; Fung et al., 2018; Catapano et al., 2019). In this procedure, the surgeons co-apt the patients’ sural nerve graft to the supratrochlear or other healthy donor nerve, contralateral to the NK cornea. The sural autograft is subcutaneously tunnelled into the patients’ affected eye and separated into
fascicles which are then sutured directly into the corneal stroma (Elbaz et al., 2014; Bains et al., 2015; Fung et al., 2018; Catapano et al., 2019). Although the modified procedure involves harvesting the sural nerve graft from the patients' lower leg, sural nerve graft harvest was associated with minimal morbidity in both adults (Miloro and Stoner, 2005; Hallgren et al., 2013) and paediatric patients (Lapid et al., 2007). Upon evaluation of 19 eyes that underwent the modified indirect corneal neurotization, there was a significant improvement in corneal sensation 24 +/- 16.1 (mean +/- SD) months post-operatively.

Figure 1-12 Corneal neurotization. End-to-end nerve repair is performed. The patient’s sural nerve autograft is attached to the supratrochlear nerve. The graft is separated into fascicles, and the fascicles are sutured around the NK affected cornea. Illustrated by Farheen Ali.

These patients also demonstrated an improved best corrected visual acuity (BCVA) and corneal epithelial health (Catapano et al., 2019). In another study, our group demonstrated reinnervation of the cornea after corneal neurotization in NK patients using in vivo confocal microscopy (Figure 1-13) (Fung et al., 2018). The indirect corneal neurotization technique developed by Borschel, Zuker, and Ali has been adopted by several centers across the world (Ting et al., 2018; Weis et al., 2018). Other variations of
indirect corneal neurotization involve the use of great auricular nerve grafts (Benkhatar et al., 2018; Jowett and Pineda, 2018) and cadaveric acellular nerve allografts (Leyngold et al., 2019) have been reported.

**Figure 1-13 In vivo confocal microscopy of subbasal nerves before and after corneal neurotization**

A. Patient’s cornea six months before corneal neurotization and B. corresponding in vivo confocal microscopy image with absent corneal nerves in subbasal cornea. C. Patient’s cornea six months after corneal neurotization, arrows pointing to nerve fascicles from autograft and D. corresponding in vivo confocal microscopy image demonstrating re-innervating axons in subbasal corneal layer.

(Reproduced from Fung et al, 2018 with permission from Elsevier: License #4595690930627)
Variable improvement in post-operative BCVA was documented in most reports of corneal neurotization. Leyngold et al (2019) reported a significant BCVA improvement from 20/70 to 20/20 in one case. However, this patient underwent the procedure nine months after NK diagnosis before incidence of corneal scarring (Leyngold et al., 2019). In comparison, patients treated by other groups were diagnosed with NK at least five years prior to corneal neurotization and many presented with PEDs and scarring pre-operatively (Benkhatar et al., 2018; Jowett and Pineda, 2018; Catapano et al., 2019). Although Leynold et al (2019) demonstrated success with the use of an acellular nerve allograft in this case-report, nerve autografts are the gold standard for nerve reconstruction following nerve damage (Patel et al., 2018). Terzis et al (2009) reported only one of six patients presenting with a high corneal sensation post-operatively measured by Cochet-Bonnet esthesiometry, and low corneal sensation (<20 mm) in two patients. In contrast, Catapano et al (2019) found that 11 and 13 of 19 eyes presented with high central and peripheral corneal sensation, respectively. Only one patient in this study presented with low corneal sensation post-operatively. The more efficient indirect re-innervation by Borschel, Zuker and Ali, compared to direct corneal neurotization, may be due to patient age, surgical technique and the number of axons in the donor autograft. Although promising, about 15% of patients treated did not reach levels of what is considered protective corneal sensation (>20 mm) and continue to suffer from NK symptoms (Catapano et al., 2019).

IV - ANIMAL MODELS OF CORNEAL REINNERVATION AND NEUROTIZATION

Previous animal models have exclusively focussed on corneal reinnervation from native corneal nerves. These models investigated corneal re-innervation in HSV-1 keratitis (Martin, 1996; Lambiase et al., 2008), diabetic keratopathy (Chikamoto et al., 2009; Yin et al., 2011), lamellar flap surgery (Namavari et al., 2011; Chaudhary et al., 2012), corneal abrasion (Li et al., 2011), and corneal transplantation (Omoto et al., 2012). Infectious and metabolic diseases such as HSV-1 keratitis (Martin 1996; Lambiase et al. 2008), diabetic keratopathy (Chikamoto et al., 2009; Yin et al., 2011) result in diffuse damage to corneal innervation. However, a portion of corneal nerves remain intact. Damage to portions of
corneal innervation can also occur due to lamellar flap surgery (Namavari et al., 2011; Chaudhary et al., 2012), corneal abrasion (Li et al., 2011), and corneal transplantation (Omoto et al., 2012). Partially intact corneal innervation provides a potential source of corneal reinnervation and thus, these reinnervation models are not applicable in models of complete corneal denervation.

Catapano et al (2018) developed the first and only animal model of corneal neurotization using a donor nerve (Figure 1-14), modelled after the technique in NK patients (Elbaz et al., 2014; Bains et al., 2015). In the rat model, corneal denervation was achieved by stereotactic ablation of the ophthalmomaxillary nerve (V1), and sural and common peroneal (CP) nerve autografts were used to re-innervate the denervated cornea. The procedure significantly increased nerve fiber density in the denervated and reinnervated cornea as compared to denervation alone (J. Catapano et al., 2018) (Figure 1-15). Although normally innervated and neurotized corneas exhibited the same nerve density quantitatively, the nerve density of the neurotized cornea was not as uniform and did not display the whorl pattern of the normally innervated subbasal nerve plexus. This may be due to the differences during development and in adulthood in the corneal architecture. These include differences in the arrangement of stromal lamellae and/or the presence or absence of axonal guidance molecules. Further, in the rat NK model, only a small proportion of regenerating nerve fibers from the donor autografts reinnervated the cornea (J. Catapano et al., 2018). The sural and CP nerve autografts contain both myelinated and unmyelinated nerve fibers, thus perhaps the cornea regulates which and what number of nerves reinnervate the NK cornea. The possible reasons for differences in the pattern of corneal innervation and reinnervation, and why only a small proportion of fibers reinnervate the cornea from the grafts need to be explored further. Additionally, the reinnervated corneas were clear suggesting that the reinnervating axons were unmyelinated. The animal model of corneal neurotization provides an ideal method to study the effects of corneal reinnervation in NK and a means to understand how to improve outcomes for NK patients who have not benefited from the procedure.
Figure 1-14 Model of corneal neurotization. A. Corneal neurotization is performed through coaptation of the common peroneal and sural nerves to the infraorbital nerve in the rat. The donor autografts are guided to reinnervate the contralateral NK cornea. After the procedure, a tarsorrhaphy is performed to prevent damage to the nerve grafts and to prevent corneal ulceration in our models (Illustrated by Kasra Tajdaran). Above, B. the nerve grafts overlay the cornea, C. the nerves are then brought below the conjunctiva and sutured directly to the cornea, D. the conjunctiva is subsequently closed over the grafts to promote revascularization.
Corneal reinnervation after neurotization. Demonstration in a Thy-1 GFP+ rat that expresses a green fluorescent protein (GFP) in axons of A. the normal innervation of the cornea; B. loss of GFP positive axons four weeks after corneal denervation, and C. reinnervation of the NK cornea, four weeks after denervation. (Catapano et al., 2018)

V - WOUND HEALING AFTER CORNEAL NEUROTIZATION

There is a well-documented impairment in epithelial healing after injury in the NK cornea (Mikulec and Tanelian, 1996; Nagano et al., 2003; Nakamura et al., 2003). Many conventional NK treatments aim to potentiate corneal epithelial cell proliferation and thus healing using topical agents as discussed in Chapter 1.5.II. Several animal models of NK and diabetic keratopathy also demonstrate an impairment in corneal healing following epithelial injury (Araki et al., 1993; Xu and Yu, 2011; Ljubimov and Saghizadeh, 2015), which is improved with the use of topical agents such as NGF (Lambiase et al., 2000), SP and IGF-1 (Nagano et al., 2003; Nakamura et al., 2003), and corticosteroids (Ashton and Cook, 1951; Gasset et al., 1969; Phillips et al., 1983). Corneal neurotization in human patients has decreased the incidence of clinically evaluated PEDs, suggesting an improvement in epithelial cell proliferation and healing (Terzis et al., 2009; Catapano et al., 2019). Catapano et al. (2018) demonstrated that corneal neurotization significantly improves epithelial healing in the rat denervated cornea (Figure 1-16 A). Corneal epithelial injury/de-epithelialization was stained with fluorescein, which was observed under a UV Wood’s lamp. Four days after de-epithelialization over the entire cornea, corneal neurotization completely re-epithelialized the denervated cornea, mirroring the healing observed in normally innervated corneas. In contrast, complete re-epithelialization of the denervated cornea was absent in the untreated cornea (J. Catapano et al., 2018) (Figure 1-16 B). The marked improvement in corneal wound healing in our NK rat model that received the corneal neurotization, was attributed to the re-innervating axons of the graft. However, the mechanism(s) by which corneal reinnervation influences epithelial health and proliferation after injury, remains unknown.
Figure 1-16 Corneal healing in the denervated cornea is improved with corneal neurotization. A. Corneal neurotization (middle row) improved corneal healing over time in the NK cornea compared to corneal denervation alone, shown in the bottom row. B. Area of corneal healing was quantitatively assessed and there was a significant improvement in healing in the neurotized group compared to the denervated group four days after corneal epithelial debridement. (Catapano et al., 2018).
1.6 Thesis Aims and Hypothesis

The primary aim of this thesis is to investigate the effect of corneal neurotization (corneal reinnervation using peripheral nerve autografts) on corneal epithelial maintenance of a denervated cornea in a model of neurotrophic keratopathy (NK). Corneal neurotization is a surgical procedure that has restored corneal sensation and innervation in the majority of NK patients, but, 15% of patients undergoing the procedure failed to reach levels of protective corneal sensation (Catapano et al., 2019). Our laboratory has developed a rat model of NK and corneal neurotization which can be used to better understand the effects of the procedure. Using the rat models, the aims of the experiments described in Chapter 2 of this thesis are:

AIM 1 – To investigate the effect of corneal neurotization on corneal epithelial and stromal thickness of a denervated cornea in a rat model of NK.

Rationale. The cornea is responsible for two thirds of the refraction of light that passes through it. Hence, maintaining and/or restoring corneal thickness is important for visual acuity. In NK, there is thinning of the corneal epithelium (Alper, 1975; Ferrari et al., 2011) due to an impairment of epithelial cell proliferation, migration and adhesion (Sigelman and Friedenwald, 1954; Müller et al., 2003). Clinically, NK can be associated with stromal inflammation as well as stromal ulceration (melting) and perforation (Dua et al., 2018). However, the effect of denervation on the stroma and stromal thickness is not well studied. Stromal thickness in the absence of corneal abrasion (protected by a tarsorrhaphy) has not yet been evaluated in corneal denervation or corneal reinnervation.

Hypothesis. Corneal neurotization prevents or reverses corneal epithelial thinning and stromal thickness abnormalities in the denervated cornea.

AIM 2 – To investigate the effect of corneal neurotization on epithelial ulceration, and corneal scarring and perforation after corneal denervation in the same rat model of NK.
Rationale. Impairment of epithelial cell proliferation (Reid et al. 1993; Garcia-Hirschfeld et al., 1994), migration (Mikulec and Tanelian, 1996; Nakamura, Nishida, et al., 1997; Chikama et al., 1998) and adhesion (Araki et al., 1993; Ko et al., 2009) leads to more severe, and characteristic symptoms of NK in patients. These include corneal epithelial breakdown or ulceration and progressive corneal scarring. In some cases, NK can result in perforations of the cornea (Semeraro et al., 2014; Mastropasqua et al., 2017). The question remains as to whether reinnervation by peripheral nerves can reverse these changes.

Hypothesis. Corneal neurotization protects the denervated cornea from epithelial ulceration, scarring, and perforation.

AIM 3 – To evaluate if, in the denervated cornea, nerve-derived peptides Substance P (SP) and calcitonin gene-related peptide (CGRP) are restored after corneal neurotization.

Rationale. Nerve-derived peptides SP and CGRP are critical in maintaining the integrity of the corneal epithelium by promoting corneal epithelial cell proliferation, migration, and adhesion in the cornea (Sigelman and Friedenwald, 1954; Müller et al., 2003). Subcutaneous capsaicin injection, that blocks SP function, in mice results in typical symptoms of NK, including corneal opacification (Fujita et al., 1984; Shimizu et al., 1987). Furthermore, concentrations of nerve-derived peptides are decreased in tears of patients with reduced corneal sensation (Yamada et al., 2000).

Hypothesis. Following corneal neurotization, the reinnervating axons restore the nerve-derived peptides, SP and CGRP, to the denervated cornea.
Chapter 2. V and 2.3.II is modified from the following:

2.1 INTRODUCTION

Corneal sensory innervation preserves corneal transparency and shape, allowing for transmission of light to the retina for visual processing. Corneal sensory nerves not only protect the corneal surface through blinking and tearing reflexes, but also release trophic factors that maintain corneal integrity and aid in corneal healing after injury. In the absence of sensory innervation of the cornea, patients develop neurotrophic keratopathy (NK). This disease is characterized by corneal epithelial breakdown (ulceration), progressive scarring and eventual vision loss (Bonini et al., 2003; Ramaesh et al., 2007; Sacchetti and Lambiase 2014). Standard treatments with topical agents and protective lenses (Sacchetti and Lambiase, 2014; Semeraro et al., 2014; Mastropasqua et al., 2017; Versura et al., 2018), fail to treat the underlying cause of NK, namely the absence of protective corneal sensation.

The absence of innervation in NK leads to epithelial cell abnormalities and failure of the cornea to protect itself from the effects of trauma, drying and further infection. The corneal epithelium is the most superficial layer of the cornea, and thus forms a protective barrier against the external environment. Maintenance of corneal epithelial thickness and corneal clarity is essential for normal vision. In both animal models and patients, NK results in corneal epithelial thinning and ulceration, and can lead to eventual corneal scarring and vision loss (Alper, 1975; Semeraro et al., 2014; Mastropasqua et al., 2017). The thinning of the corneal epithelium in animal and patients appears to be the result of impaired corneal epithelial adhesion and proliferation during normal epithelial turnover, demonstrated in animal models (Sigelman and Friedenwald, 1954; Alper, 1975). This impairment is presumably due to loss of nerve-derived trophic support in NK (reviewed by Sigelman and Friedenwald 1954). Epithelial thinning affects the refraction of the light passing through the cornea, resulting in lower visual acuity (Reinstein et al., 2008). Additionally, the morphology of corneal epithelial cells is altered in the rabbit denervated corneas with the cells exhibiting swelling (Gilbard and Rossi, 1990) and, in the surface epithelial cells, the loss of microvilli (Alper, 1975). It is important to note that the
morphology of corneal epithelial cells may be different between species when translating information learned from animal models pertaining to humans (Henriksson et al., 2009).

Epithelial cell loss and abnormalities lead to diminished release of epithelial cell-derived soluble factors, which are involved in stromal healing and remodeling in response to injury or infection (Sacchetti and Lambiase, 2017). In the normally innervated corneal stroma, nerve fibers travel parallel to stromal lamellae, the precise organization of which is essential for corneal clarity (Maurice, 1957, 1970; Jester et al., 1999; Hassell and Birk, 2010), before branching to eventually terminate in the corneal epithelium. A minority of corneal nerve fiber endings are found within the stroma. Some of these fibers terminate in close proximity to keratocytes, specialized fibroblasts involved in stromal healing after injury (Muller et al., 1996; Seyed-Razavi et al., 2014). Corneal infection and injury can increase stromal ulceration (melting), and can lead to stromal scarring and perforation. This can result in corneal opacification and eventually corneal blindness (Reviewed by Dua et al., 2018). In severe cases, corneal perforations are treated with cyanoacrylate glue (Fogle, Kenyon and Foster, 1980; Semeraro et al., 2014), conjunctival flaps (Gundersen and Pearlson, 1969), or keratoplasty (Jhanji et al., 2011; Lambley et al., 2014).

Substance P (SP) and calcitonin gene-related peptide (CGRP) are co-localized with axons in the cornea. The initial impairment in epithelial cell proliferation (Reid et al. 1993; Garcia-Hirschfeld et al., 1994), migration (Mikulec and Tanelian, 1996; Nakamura, Nishida, et al., 1997; Chikama et al., 1998) and adhesion (Araki et al., 1993; Ko, Yanai and Nishida, 2009), that leads to more severe symptoms of NK in animal models, has been attributed to loss of these nerve-derived peptide factors the functions of which have been confirmed in vitro. NK treatments in patients have, in the past, focused on restoring trophic support through the use of these peptides administered topically in combination or separately (Brown et al., 1997; Chikama et al., 1998; Yamada et al., 2008). A recently developed surgical technique of corneal neurotization by a peripheral nerve via implantation of an autograft in NK patients restored innervation in the corneas of NK patients with resulting increased sensation and improved corneal health. (Elbaz et al.,
2014; Bains et al., 2015; Fung et al., 2018; Catapano et al., 2019). However, a small percentage of patients fail to reach standards for normal corneal sensation (Catapano et al. 2019).

In this study, we asked the question of whether corneal neurotization first, restores the nerve-derived peptides SP and CGRP in the denervated cornea as a model of NK, and second, whether it improves corneal epithelial integrity. We used our recently developed rat model of NK, in which the cornea is denervated by transection of the ophthalmomaxillary nerve, and of corneal neurotization in which the denervated cornea is reinnervated by the contralateral infraorbital nerve via a peripheral nerve autograft. We used hematoxylin and eosin staining of corneal sections to compare epithelial and stromal thickness in denervated, reinnervated and normally innervated corneas. Fluorescein staining was used to assess the extent of ulceration under UV light, and scarring and perforation were assessed under normal light. Third, the presence of SP and CGRP in the cornea of the three groups was determined and measured with immunohistochemistry and Western blotting, respectively. Our findings demonstrate that corneal neurotization promotes epithelial integrity in the denervated NK cornea by preventing epithelial thinning, ulceration, scarring and perforation. Furthermore, the re-innervating axons restore SP and CGRP in the denervated cornea.

2.2 MATERIALS AND METHODS

1 – Animals
Female Sprague Dawley rats (250-350g) were used in all experiments. All experiments were approved by The Hospital for Sick Children Laboratory Animal Services and adhered to the guidelines of the Canadian Council on Animal Care as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were maintained in a 12:12 hour light:dark cycle in a temperature- and humidity-controlled environment. Rats received ad libitum water and standard rat chow (Purina, Mississauga, ON, Canada).
II – Sample size and group distribution

Rats (total n = 45) were split into three groups: one control group (normally innervated cornea) and two experimental groups i) corneal denervation (NK model) and ii) corneal denervation + corneal neurotization (Table 2-1). Seven rats per group (n=21) were used for evaluation of corneal epithelial and stromal thickness. Corneal sections from these same rats were used for immunostaining. Three additional rats from each group (n=9) were used for Western Blot analysis. Five rats with only corneal denervation, and 10 that received corneal neurotization were assessed for corneal epithelial ulceration using fluorescein staining.

<table>
<thead>
<tr>
<th></th>
<th>Normally innervated cornea (control)</th>
<th>Corneal denervation (NK model, negative control)</th>
<th>Corneal denervation + corneal neurotization</th>
<th>n/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>V – Evaluation of corneal epithelial and stromal thickness</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>VI – Evaluation of corneal ulceration, scarring and perforation</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>VII – Immunohistochemistry</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>(biological replicates of IV)</td>
</tr>
<tr>
<td>VIII – Western blot</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Total n</td>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>

Table 2-1. Sample size & group distribution
II – Sterile surgeries
Surgical procedures were performed in an aseptic manner using an operating microscope (Leitz, Willowdale, ON, Canada). Rats were operated on under inhalational anesthetic (2% isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ, USA) and given metacam (2 mg/kg; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) for pain relief. At the final outcomes assessment, rats were euthanized under deep anesthesia using intraperitoneal Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg; Bimeda-MTC, Cambridge, ON, Canada).

Figure 2-1 Rat models. In our rat model, A. denervation of the left cornea was achieved with stereotactic ablation of the left ophthalmomaxillary nerve (V1), and B. corneal neurotization was achieved by coapting common peroneal (CP) and sural nerve autografts to the right infraorbital nerve and implanting the grafts into the left cornea (illustrated by Kasra Tajdaran).

Corneal denervation and corneal neurotization in a rat model of NK was described in detail previously (Joseph Catapano et al., 2018). Briefly, denervation of the left cornea was achieved by stereotactic ablation of the left ophthalmomaxillary nerve (V1) (Figure 2-1A). This ablation was performed using a modified insulated 22-G monopolar electrode (UP 3/50; Pajunk GmbH, Geisingen, Germany), in which 1mm of the insulation at the
electrode tip was removed with a scalpel prior to the ablation surgery. In surgery, rats were mounted onto a stereotactic frame (Harvard Apparatus, Hollingston, MA, USA). A midline cranial incision was made to expose bregma. Bregma is the location where the sagittal and coronal sutures of the skull cross, from which the coordinates of V1 were calculated. A 1 mm burr-hole was made through the skull with a drill 2.0 mm anterior and 1.5 mm lateral (left) to bregma. The modified electrode was then lowered to a depth of 10 mm through the burr hole, such that the non-insulated portion of the electrode pierced through V1. Subsequently, the ophthalmomaxillary nerve was ablated (10 W for 60 seconds) using an electrosurgical generator (Force FC-8C; Medtronic, Fridley, MN, USA). The electrode was then removed, and the skin incision sutured. Ablation was confirmed with observation of a blown pupil, absent blink reflex and absent response to suturing of the eyelid. The coordinates of V1 were confirmed after sacrifice.

Corneal neurotization was performed under an operating microscope (Figure 2-1B). A skin incision made connecting the greater trochanter and knee joint, as well as the knee and ankle joint. Thereafter the biceps femoris muscle was separated from the vastus lateralis muscle to expose, dissect, and harvest ~30 mm of the common peroneal (CP) nerve. The gastrocnemius muscle was freed from the tibialis anterior muscle to expose, dissect and harvest the same length of the sural nerve. The nerves were each placed on a saline soaked gauze until the coaptation of the two nerves to the proximal stump of the transected right infraorbital nerve and tunneled subcutaneously to the left cornea (Fig. 2-1B). The latter nerve was exposed an incision that was made parallel to the right contralateral whisker pad and the nerve branches to the whisker pad were identified. Two branches were transected and the proximal stump of each cross-sutured to the CP and to the sural autografts. The autografts were tunneled under the conjunctiva and loosely sutured onto the corneoscleral junction at the 12 and 6 o’clock position with 9-0 vicryl. Finally, the conjunctiva was closed over the grafts to promote revascularization.

A protective tarsorrhaphy, suture of the eyelids, was applied after each corneal neurotization and denervation surgery to prevent damage to the cornea after corneal denervation and to protect the autografts following corneal neurotization.
**Timeline.** In the treatment group, corneal neurotization was performed six weeks prior to the first corneal denervation to allow sufficient time for the axons to grow through the grafts. In the neurotized and NK groups, the second denervation was performed three weeks after the first to prevent native corneal nerves from re-innervating the cornea. Subsequent assessments were performed beginning four weeks after initial corneal denervation, (Figure 2-2).

**Figure 2-2 Experiment timeline.** Timing of corneal neurotization, denervation, tarsorrhaphy removal and corneal harvest represented on timeline. Experiments to which these procedures belong are represented by the assigned roman numerals within the Material and Methods section (V – epithelial and stromal thickness; VI – ulceration, scarring and perforation; VII – immunohistochemistry; VIII – western blot). Rat groups are denoted by colour and number (figure legend in top right corner). In group 1, corneal neurotization is performed 6 weeks prior to corneal denervation. In groups 1 and 2, corneal denervation is performed twice, 3 weeks apart. One week after the second denervation the affected corneas are harvested with the exception of the rats undergoing observation of the ocular surface for ulceration (VI) in which corneas are harvested one week later.
IV – Corneal harvest

At the time of harvest, eyes were enucleated and whole-globes were fixed in 4% paraformaldehyde (PFA) and 0.2% saturated picric acid in 1X Phosphate Buffer Solution (PBS) for 15 minutes over ice. The cornea and limbus were then dissected and fixed for another 45 minutes over ice. Following fixation, corneas were placed in 30% sucrose, 1X PBS overnight in 4 °C. Corneas were embedded in OCT, kept in a -80 °C freezer, and cut in 10 μm cross-sections onto gel-coated slides (Fisherbrand Superfrost Plus Microscope slides) using a cryostat (Leica CM3050).

V – Evaluation of corneal epithelial and stromal thickness

Denervated corneas and those denervated corneas which were neurotized (n=7 per group) were harvested four weeks after the initial V1 ablation (Figure 2-2 A). Normal unoperated and innervated corneas (n=7) were harvested at the same timepoint. Three 10 μm cross-sections from each cornea were sampled from representative areas of the cornea and stained with Hematoxylin and Eosin. Sections were subsequently imaged with bright-field microscopy under a 20X objective (overall 200X magnification; Leica). The central and peripheral epithelial and central stromal thicknesses of each section were measured using the computer program ImagePro.

VI – Evaluation of corneal ulceration, scarring and perforation

Four weeks after the initial V1 ablation, the tarsorrhaphy was removed in all rats exposing the denervated cornea (Figure 2-2 B, D). The denervated corneas in the rats that did not receive treatment (negative control, n = 5) were compared to those in which corneal neurotization was performed (treatment group, n = 10). Standardized digital photographs (Nikon D 5100; Nikon, Tokyo, Japan) of the corneas were taken daily for one week. To keep the camera at a fixed distance from the cornea, imaging was performed using a standardized frame. The photographs were obtained under i) normal light and ii) with a Wood's lamp/fluorescein staining (DioFluor Strips; Innova Medical Ophthalmics, Inc., Toronto, Canada) to assess corneal scarring and epithelial breakdown, respectively. Perforation was assessed by in vivo observation of a hole in the cornea. Incidence of ulceration and perforation was considered a binary outcome that was compared between
experimental groups using a Fisher’s exact test. The area of de-epithelialization was calculated as a percentage of the entire cornea using the computer program ImageJ.

VII - Immunohistochemistry

Slides were permeabilized with methanol for 10 minutes at -20 °C. Slides were subsequently washed with 1X PBS at room temperature (RT). Antigen retrieval was performed using a citrate buffer solution (Sigma Aldrich) that was boiled in a microwave. Slides were put into solution and brought back to a boil before cooling over ice to RT. The boil and re-cooling were repeated. Slides were then washed in 1X PBS and subsequently incubated for two nights with primary antibody (See Table 1) diluted with 1X PBS with 0.1% Triton X and 5% serum in 4 °C. Control corneal sections were incubated with the aforementioned diluting solution without primary antibodies. Slides were washed with 1X PBS, incubated with secondary antibodies (See Table 1) in 1X PBS with 0.1% Triton X and 5% serum in RT for 30 min. Subsequently slides were washed at RT and mounted. Slides were imaged using Zeiss AxioVision 4.8.2 software under a fluorescent Zeiss Axioplan 2 upright microscope (Toronto, ON) with a Hamamatsu ORCA-R2 C10600 camera (Bridgewater, NJ) at 20X magnification.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Host</th>
<th>Company</th>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P (SP)</td>
<td>1:50</td>
<td>Mouse</td>
<td>Santa-Cruz Biotechnology</td>
<td>Cy3</td>
<td>1:1000</td>
<td>Jackson Immuno</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide (CGRP)</td>
<td>1:50</td>
<td>Rabbit</td>
<td>Sigma-Aldrich</td>
<td>anti-biotin</td>
<td>1:1000</td>
<td>Jackson Immuno</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Streptavidin-Cy5</td>
<td>1:500</td>
<td>Jackson Immuno</td>
</tr>
<tr>
<td>β-iii Tubulin</td>
<td>1:300</td>
<td>Chicken</td>
<td>Abcam</td>
<td>488</td>
<td>1:1000</td>
<td>Jackson Immuno</td>
</tr>
</tbody>
</table>

Table 2-2. Antibody solutions and dilutions used in thin-section and whole-mount immunohistochemistry
VIII - Western blot
For immunoblotting analysis of Substance P, three corneas were harvested from each group four weeks after initial denervation (Figure 2-2 A). After washing twice with 1X PBS, the corneas were homogenized with 300 µl of lysis buffer (150 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% Sodium dodecyl sulphate, 1% Nonide P-40, 0.5% Sodium Deoxycholate, 10% protease inhibitors) on ice. After centrifuging at 10,000 rpm for 30 min at 4 °C, the supernatant was collected.

Protein concentration was measured using a BCA protein assay kit [Bovine Serum Albumin (BSA) set, Thermo Fisher Scientific, cat. no. 23208] with BSA as the standard. Tissue lysates (40 µg for each sample) were resolved on 10% SDS-PAGE mini electrophoretic gels. Following SDS-PAGE, the protein was transferred onto PVDF membrane (Immu-No-Blot PVDF membranes, Bio-Rad Laboratory, cat. No. 162-0177) and subjected to electrophoresis for 1.5 hours at RT. The proteins were blocked with a 3% of BSA in Tris-buffered saline with Tween 20 (TBST), incubated with the primary antibodies (anti-Substance P antibody, Santa Cruz, cat. no. sc-9758, 1:100; anti-GAPDH, Sigma, cat. no. G9545, 1:3000) at 4 °C overnight, and incubated with a secondary antibody that was conjugated to horseradish peroxide for 1 hour at RT.

For semi-quantitation of protein, the VDF membranes were incubated in an enhanced chemiluminescence-detection reagent (Pierce ECL Western blotting substrate, Thermo Fisher Scientific, cat. no. 32209) and the chemiluminescent signals were captured using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, cat. no. 1708290).

IX – Statistics
Statistical analysis was performed using GraphPad Prism version 8.0 for Mac (GraphPad Software, Inc., San Diego, CA, USA). One Way ANOVAs were used to compare the three groups with regard to epithelial and stromal thickness. The mean ± standard deviation (SD) area of de-epithelialization in each group was calculated daily, and means were compared at each time point using a Student’s t-test. Fisher’s exact tests and unpaired t-
tests were used to compare the denervated and neurotized corneas with respect to ulceration and perforation. Statistical significance was accepted as $p<0.05$, and all data were expressed using mean $\pm$ SD.

2.3 RESULTS

1 - Corneal neurotization prevents central epithelial thinning in the denervated corneal model of NK

Four weeks after the initial denervation of the cornea and just prior to globe enucleation, corneal dissection and fixation, the protective tarsorrhaphy was removed (Figure 2-2 A). Thinning of the central epithelium of the denervated cornea as compared to the normally innervated cornea and the recovery of thickness after neurotization are apparent from representative images shown in Figure 2-3 A. In contrast, this thinning and its recovery are not apparent in the epithelium of the peripheral cornea (Figure 2-3 A). Measurements of thickness revealed significant thinning after corneal denervation, the mean ($\pm$ SD) of the central corneal epithelium in the denervated cornea (negative control) being significantly thinner than that of the uninjured group (positive control, $10.84 \pm 1.37 \, \mu m$ vs. $16.19 \pm 1.34 \, \mu m$, $p < 0.01$) and thinner than the central cornea in the neurotized treatment group of rats ($10.84 \pm 1.37 \, \mu m$ vs. $15.08 \pm 0.42 \, \mu m$, $p < 0.01$). The corneal reinnervation by implanted sural and CP nerve autografts in the neurotized treatment group resulted in the corneal epithelial thickness equaling that in the uninjured control group, there being no significant difference between the thicknesses of $15.08 \pm 0.42 \, \mu m$ and $16.19 \pm 1.34 \, \mu m$ ($p>0.05$), in the treatment and uninjured groups, respectively. Hence, the corneal neurotization procedure either prevents central epithelial thinning and/or restores thickness in the reinnervated cornea (Figure 2-3 B).

The central epithelium in the normally innervated cornea of the rat is significantly thicker than the peripheral corneal epithelium ($16.19 \pm 1.34 \, \mu m$ vs $12.11 \pm 0.49 \, \mu m$, $p<0.01$). After denervation, there was no significant difference between the central and peripheral cornea due to the central epithelial thinning in the denervated cornea ($10.84 \pm 1.37 \, \mu m$ vs $11.26 \pm 0.93 \, \mu m$, $p>0.05$). The relationship between the healthy central and peripheral
epithelial thickness is restored in the reinnervated cornea after corneal neurotization (15.08 ± 0.42 μm vs 12.25 ± 1.48 μm, p<0.01) (Figure 2-3 B). There was no difference (p>0.05) in the mean (± SD) central stromal thickness between the neurotized and normally innervated (38.66 ± 2.29 μm) nor denervated (39.37 ± 10.10 μm) corneas. The central stroma of the denervated cornea was thinner than that of the normally innervated cornea (p<0.05) (Figure 2-3 C). Peripheral stromal thickness could not be accurately measured due to fragility of corneal sections and consequently tissue sample loss.
Figure 2-3 Corneal epithelial and stromal thickness after corneal neurotization. A. Representative 1X1 images of the central and peripheral cornea stained with Hematoxylin & Eosin in the three groups of rats in which the corneas were normally innervated, denervated and neurotized corneas. Scale bars 20 μm B. The central epithelial thickness in the denervated cornea (black C) was significantly thinner than that of the normally innervated cornea (clear C; p<0.01) and neurotized cornea (blue C; p<0.01). There were no differences between peripheral (P) epithelial thicknesses in any of the three groups. C. There was a difference between the central stromal thickness of the normally innervated and denervated corneas. There were no differences in the central stromal thickness between the neurotized corneas (blue) and the normally innervated corneas (clear) or the denervated corneas (black). In B and C, the mean values ± SD are shown with individual data points.

II - Corneal neurotization protects the denervated cornea from ulceration, scarring and perforation

Seven days after removal of the protective tarsorrhaphy (Figure 2-2), the cornea after corneal neurotization had significantly less corneal epithelial breakdown than those of the denervated negative control cornea (0.0 ± 0.0 vs. 30.1% ± 12.7, p < 0.01) (Figure 2-4 A, B). All denervated rat corneas developed progressive epithelial breakdown, while the denervated corneas that received neurotization in only two rats developed an ulcer that healed within one week (p < 0.01) (Figure 2-4 C). Hence, the neurotization protects the denervated cornea from epithelial breakdown. Corneal neurotization reduced corneal scarring (Figure 2-5 A), and no corneas developed a perforation after neurotization as compared to the 80% of corneas that did in the untreated rat group (p < 0.05) (Figure 2-5 B). The corneal neurotization procedure thus prevents scarring and perforation in the rat model of corneal NK.
Figure 2-4 Corneal ulceration after corneal neurotization. A. Fluorescein staining (green) of the cornea between 0 and 7 days shows corneal epithelial ulceration in the denervated and neurotized groups. B. There was a significant time-dependent increase in the % of the cornea that was ulcerated in the denervated (black) group compared to the neurotized (blue) group between 2 to 7 days. C. Corneal neurotization significantly reduced incidence of corneal ulceration in the denervated cornea.

* $p < 0.05$

- Denervation (n=5)
- Neurotization (n=10)
Corneal neurotization restores SP and CGRP in the denervated cornea

Immunohistochemical staining of corneal thin-sections showed co-localization of SP and CGRP with the axons innervating the cornea, both in the normally innervated cornea (Fig 2-6 A) and the denervated cornea that were reinnervated by corneal neurotization (Fig 2-6 C). No staining was observed in the untreated denervated cornea (Figure 2-6 B). There was no staining of SP or CGRP under the control condition of incubation with only the secondary and no primary antibody incubation (Figure 2-7). Western blot analysis confirmed the absence of SP after denervation and presence in the neurotized cornea (Figure 2-8). These findings indicate that SP and CGRP from reinnervating infraorbital nerves may be responsible, at least in part, for the proliferation, migration, and adhesion of epithelial cells in the cornea.

Figure 2-5 Corneal scarring and perforation after corneal neurotization. A. Representative images of the same cornea in each group over seven days after tarsorrhaphy removal. Corneal neurotization protected the denervated cornea from scarring (4 days) and perforation (7 days). B. Corneal neurotization completely prevented the denervated cornea from perforating.
**Figure 2-6 SP and CGRP immunostaining.**

Immunohistochemical staining of i. β-3 tubulin (green), ii. Substance P (SP) (red), iii. Calcitonin gene-related peptide (CGRP) (purple), iv. merged in the A normally innervated (n=1), B denervated (n=1) and C. neurotized (n=1) corneas. White arrows point to true staining, not artifact. A and C demonstrate co-localization of SP and CGRP with axon fibers in the normally innervated and neurotized corneas, whereas in B there is an absence of staining in the denervated cornea.
**Figure 2-7 Control immunostaining.** No primary antibody incubation resulted in no staining of β-3 tubulin (green), SP (red), or CGRP (purple) in the A. normally innervated (n=1), B denervated (n=1), and C denervated + neurotized (n=1) corneas at 20X magnification.

**Figure 2-8 SP western blot analysis.** Corneal neurotization restores Substance P (SP) in the denervated cornea. There is no SP detected in the untreated denervated (NK) cornea. (n=3 per group)
2.4 DISCUSSION

In this study, we demonstrated for the first time that corneal neurotization in an animal model improves the epithelial integrity of the denervated cornea, possibly by restoring trophic support of nerve-derived neuropeptides. Specifically, we showed that 1) corneal neurotization protects the denervated cornea from epithelial thinning, ulceration, scarring and perforation, and 2) axons from the common peroneal (CP) and sural nerve autografts reinnervate the denervated cornea with nerve-derived trophic factors, substance P (SP) and calcitonin-gene related peptide (CGRP).

The animal model that was used in these experiments paralleled the corneal neurotization procedure that has been used successfully in human neurotrophic keratopathy (NK). Unlike the human surgery, we used two branches of the donor infraorbital nerve to cross-suture to the CP and sural nerve autografts that were tunneled and sutured to the denervated cornea. The rat surgical procedure mimicked quite closely the human surgery because the two fascicles of the infraorbital nerves that were used to reinnervate the rat cornea that is smaller than the human cornea, likely were sufficient to mimic the splaying of several fascicles of the dissected sural nerve autograft in the human cornea.

Prior to discussing our findings, it must be recognized that our experimental conditions of denervation and reinnervation in the rat are somewhat artificial. They were used because of the rapid spontaneous reinnervation of the denervated cornea (within 3 weeks) by nerve fibers from the ablated ophthalmomaxillary nerve (Catapano et al., 2018) whilst infraorbital nerve fibers were regenerating from the contralateral side to the denervated cornea. The ophthalmomaxillary nerve was ablated twice (Figure 2.1), three weeks apart before harvesting a denervated cornea seven days later to assess epithelial and stromal thinning and immunohistochemical detection of neuropeptides (Figure 2.6). For assessment of corneal ulceration, scarring, and perforation, the tarsorrhaphy was removed to open the eyelid at the same seven-day timepoint after the second ablation and the assessment performed daily for zero to seven days. For observations and measurements from reinnervated cornea, the cross-suture of two branches of the donor
infraorbital nerve to reinnervate the contralateral cornea via the CP and sural nerve autografts, was performed 6 weeks prior to the first and second ophthalmomaxillary nerve ablations described for the corneal denervation. Thus, this model is most representative of patients receiving corneal neurotization who are diagnosed before extensive epithelial ulceration and scarring, or patients who can expect corneal anesthesia secondary to an intracranial surgery, for example.

Epithelial thinning was demonstrated in the denervated cornea with Hematoxylin and Eosin staining (Figure 2-3). This finding concurred with similar findings in several animal models of corneal denervation (Reid et al. 1993; Garcia-Hirschfeld et al., 1994; Ko et al., 2009) as well as in the insensate cornea in human NK patients (Sigelman and Friedenwald, 1954). In animal studies, Hematoxylin and Eosin was similarly used to assess thinning, and in human studies, the thinning was detected using in vivo confocal microscopy and optical coherence tomography (Villani et al. 2014; Mastropasqua et al. 2018). It is suggested that the thinning is due to impaired adhesion and proliferation of corneal epithelial cells. This conjecture was based on the coincidence of the impairment with epithelial thinning (Reid et al. 1993; Garcia-Hirschfeld et al., 1994; Ko et al., 2009). Whatever the basis for the thinning, our findings of normal epithelial thickness in longitudinal sections of reinnervated rat cornea (Figure 2-3) demonstrate unequivocally the maintenance of epithelial thickness and/or the reversal of denervation-induced thinning by peripheral nerve reinnervation. Though epithelial thickness was not assessed in human patients with and without corneal neurotization, reduced persistent epithelial defects in the majority of NK patients treated with corneal reinnervation (Catapano et al., 2019) support this finding.

A significant reduction in stromal thickness was detected in the denervated cornea for the first time, using Hematoxylin and Eosin staining, indicating that corneal innervation is essential to maintain the stroma. This thinning occurred despite protection from external abrasion by a protective tarsorrhaphy. However, the stromal thickness in the reinnervated cornea, was not different from either the intact or denervated cornea, possibly due to the scatter in the measurements (Figure. 2.3C). This may be reduced by increasing the
number of observations. Additional explanations for the findings include the unintentional stromal trauma of the rat neurotization surgery and varied orientation of the corneal cross-sections relative to the autograft insertion onto the corneal surface. The latter may be addressed in future studies by measuring innervation density (Cruzat et al., 2017) concurrent with stromal thickness using confocal microscopy (Reinstein et al., 2009).

The denervated cornea that undergoes corneal ulceration, scarring and perforation, also seen in previous studies (Huang and Tseng, 1991; Araki et al., 1993; Rao et al., 2010; Blanco-Mezquita et al., 2013), was fully protected by corneal neurotization (Figures 2-4 and 2-5). As in the previous studies, the area of epithelial ulceration was measured with in vivo fluorescein staining (Huang and Tseng, 1991; Araki et al., 1993; Rao et al., 2010; Blanco-Mezquita et al., 2013). Normally fluorescein staining is not seen because the epithelial cells form a tight barrier (Lambiase et al., 1998; Matsumoto et al., 2004) which is broken down following corneal denervation. The fluorescein staining became evident as the cornea ulcerated (Figure 2-4A) and without innervation, proceeded to corneal scarring at day 4 and perforation at day 7 that was seen under the microscope with normal light (Figure 2-5A). Hence, reinnervation is essential for the preservation of normal corneal epithelium.

A proviso of our experimental method was the eyelid closure with a stitch (tarsorrhaphy) immediately after all the surgeries. The tarsorrhaphy was removed just prior to the corneal observations from zero to seven days, a week after the second denervation (Figure 2-2). The findings of the progression of ulceration, scarring and perforation are attributed to the removal of the tarsorrhaphy itself as well as the corneal denervation in contrast to the epithelial thinning (Fig. 2-3) that occurred whilst the eyelids were closed. The tarsorrhaphy was removed at day zero because vascularization in the conjunctival tissue of the eyelids may otherwise have provided the ulcerated cornea with factors to promote healing (Müller et al., 2003) and would thereby confound the results. However, the lack of protection after the first day may have hastened corneal abrasions in our rat model of corneal denervation, unlike the protection offered by lenses or protective glasses in NK patients (Donnenfeld et al., 1995; Ramaesh et al., 2007). Nonetheless, despite rapid symptom
progression in the rat untreated denervated cornea, we demonstrated that corneal neurotization prevented ulceration, scarring, and perforation. A protective cone would be efficacious in protecting the rat cornea from external abrasions in future experiments. In addition, the same ophthalmic slit lamp used by ophthalmologists in the clinic would allow assessment of the depth of the ulceration.

Our immunostaining of the reinnervating infraorbital nerve axons via the peripheral nerve autografts used in the corneal neurotization procedure demonstrated their expression of the SP and CGRP neuropeptides. These peptides were co-localized with the re-innervating axons and were absent in the untreated denervated cornea (Figure 2-6 and 7). A "no primary antibody" control was used to confirm staining, in which the tissues of interest were only incubated with the secondary antibodies, but there are several other controls that could have been used. A similar method would be to incubate the tissues of interest with only the primary and no secondary antibodies. Alternatively, a tissue that is known to express or not express the target antigen can be used as a positive or negative tissue control. For example, a positive control for the SP antibody could be the trigeminal ganglion, in which SP is produced (Lehtosalo, 1984), where we would expect true staining to appear. Conversely, any staining observed in tissue of a SP knock-out model can be would be a false-positive or non-specific. An isotype control, an antibody with the same isotype, clonality, and host-species as the primary antibody, can be used in lieu of the primary antibody to ensure the observed staining is not due to non-specific interactions of the antibody and tissue. Lastly, any staining observed in the tissue incubated with a primary antibody pre-absorbed with the immunogen of interest, SP or CGRP, would also indicate non-specific staining.

Our western blot analysis confirms that SP is restored in the denervated cornea by corneal reinnervation by neurotization. Whilst the calculated molecular weight for SP is ~13kDa, the observed molecular weight with Western blot can vary due to post-translational modifications, cleavages, charges and so forth which explains our observation of the SP band at 18kDa (Figure 2-8). To validate the band in Western blot is in fact SP, a positive and negative tissue control, such as those mentioned above for
immunohistochemistry controls, could be used. A band at the same observed molecular weight for a positive tissue control, and an absence of a band for a negative tissue control would confirm the band is indeed SP.

In the normally innervated cornea, neuropeptides, such as SP and CGRP, function to maintain epithelial cell adhesion and promote their epithelial cell proliferation and migration to the central cornea in response to the ongoing epithelial cell turnover and healing after injury (Reid et al. 1993; Garcia-Hirschfeld, Lopez-Briones, and Belmonte 1994; Mikulec and Tanelian 1996; Nakamura et al. 1997; Chikama et al. 1998; Araki et al. 1993; Ko, Yanai, and Nishida 2009). In human NK, topical applications of SP and CGRP promote healing of epithelial defects (Brown et al., 1997; Chikama et al., 1998; Yamada et al., 2008). The observed improvement in the restoration and/or maintenance of the corneal epithelium after reinnervation of the denervated cornea in our rat model of NK (Figures 2-3 to 2-5), suggests that the peptides may function similarly in neurotized NK corneas and normally innervated corneas to promote healing of epithelial defects. Future studies need to be performed to elucidate the specific mechanism(s) by which SP and CGRP promote epithelium maintenance in the neurotized cornea.

In summary, this study demonstrated that corneal neurotization restores and/or maintains epithelial integrity in the denervated rat cornea by preventing epithelial thinning, ulceration, scarring, and perforation. We hypothesize that this may be due, at least in part, to the restoration of the nerve-derived peptides, SP and CGRP, in the neurotized cornea. Our study provides further insight into why corneal neurotization is effective in preventing disease progression and maintaining corneal epithelial integrity in the majority of NK patients.
CHAPTER 3  Conclusions and Future Directions
3.1 – Summary of results

In this thesis, we have demonstrated that corneal neurotization maintains epithelial integrity of the denervated cornea in our rat model of Neurotrophic Keratopathy (NK). We have shown corneal neurotization protects the cornea from the epithelial thinning, ulceration, scarring and perforation that occurs in the denervated cornea. In denervated corneas that received corneal neurotization, nerve-derived peptides Substance P (SP) and calcitonin gene-related peptide (CGRP) were restored by the re-innervating axons from the nerve autografts, which offers a potential mechanism of improved ocular surface health.

3.2 – Future Directions

Our future directions and hypotheses derive from the results of this thesis and published literature. Due to the severity of NK and the difficulty of managing symptoms in patients, an extensive amount of research has focused on normal and impaired corneal innervation. While some groups have investigated nerve regeneration in corneal diseases associated with corneal hypoesthesia (Martin, 1996; Lambiase et al., 2008; Chikamoto et al., 2009; Yin et al., 2011), only one paper, published by our group, has evaluated the effects of re-innervating the cornea with donor nerves (Catapano et al., 2018). Previously published literature about corneal deficits following denervation in models of NK inform our future directions in investigating corneal reinnervation by nerve autografts (future directions 1, 2, 5). Little is known about the relationship between innervation, Schwann cells (SCs), and limbal stem cells (LSCs). Published literature about innervation, SCs and stem cells in other tissues as well as preliminary investigations by our laboratory inform future directions 3 and 4. These investigations focus on understanding the relationship between these cells and innervation in the normal and denervated cornea to inform investigations into neurotization’s effect on this relationship.
FUTURE DIRECTION 1 – CORNEAL EPITHELIUM
Evaluating ocular surface health is the first step to understand how corneal re-innervation influences the denervated corneal epithelium. In our rat model, corneal neurotization prevents the central corneal epithelium from the thinning, ulceration, perforation and scarring that follows corneal denervation (Ch. 2). Corneal epithelial thinning in NK is associated with changes in epithelial cell morphology. In response to corneal denervation epithelial cells demonstrate swelling in rabbits (Gilbard and Rossi, 1990) and, loss of surface cell microvilli in rats (Alper, 1975; Hosotani et al., 2006). A future direction is to investigate whether the morphological changes of the epithelial cell that typically accompany corneal denervation, remain in the reinnervated cornea. Due to the similarities between the ocular surface health in the normally innervated and denervated corneas treated with corneal neurotization, we hypothesize the neurotization procedure will preserve the normal healthy epithelial cell morphology. Immunohistochemical staining of Keratin 3/12 (K3 and K12) and CD44-positive epithelial cells can be used (Table 3-1). Alternatively, epithelial cell morphology may be assessed with electron microscopy as in other studies (Hosotani et al., 2006; Chalvatzis et al., 2012)

FUTURE DIRECTION 2 – TROPHIC SUPPORT
In this thesis, we confirmed the absence of nerve-derived peptides, SP and CGRP, in the denervated cornea and demonstrated their co-localization with the re-innervating axons of autografts used in the corneal neurotization surgical procedure. To do this we used thin-section immunohistochemistry to permit the visualization of smaller nerve segments in the cornea. We are currently performing whole-mount SP and CGRP immunohistochemical staining in the rat cornea which allows a larger number of nerve fibers to be visualized in several layers of the cornea and in continuity. However, this technique has proven to be very difficult for staining the weaker antibodies, and optimization of this technique in our laboratory is ongoing. Whilst we have characterized these nerves as containing SP and CGRP, we have not yet evaluated whether the trophic support, normally provided by these nerve-derived peptides, is restored in the neurotized cornea.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Indication of</th>
</tr>
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<tbody>
<tr>
<td>K3 (cyto-)keratin 3</td>
<td><em>Epithelial cell marker</em>&lt;br&gt;- With K12, specific for corneal epithelium</td>
</tr>
<tr>
<td>K12 (cyto-)keratin 12</td>
<td><em>Epithelial cell marker</em>&lt;br&gt;- With K3, specific for corneal epithelium</td>
</tr>
<tr>
<td>CD44 Cell surface adhesion receptor</td>
<td><em>Corneal epithelial cell marker</em>&lt;br&gt;- Receptor on corneal epithelial cell membrane</td>
</tr>
<tr>
<td>pEGFR Phosphorylated epidermal growth factor receptor</td>
<td><em>Epithelial cell proliferation</em>&lt;br&gt;- Trophic influence of SP in cornea</td>
</tr>
<tr>
<td>ZO-1</td>
<td><em>Adhesion marker</em>&lt;br&gt;- Tight junction protein&lt;br&gt;- Trophic influence of SP in cornea</td>
</tr>
<tr>
<td>E-cadherin</td>
<td><em>Adhesion marker</em>&lt;br&gt;- Tight junction protein, anchoring complex formation&lt;br&gt;- Trophic influence of SP in cornea</td>
</tr>
<tr>
<td>β-4 integrin</td>
<td><em>Adhesion marker</em></td>
</tr>
<tr>
<td>Necl4 A.K.A. SynCAM4 = Cell Adhesion Molecule 4</td>
<td><em>Schwann cell marker</em>&lt;br&gt;- Especially of those interacting with axons</td>
</tr>
<tr>
<td>GFAP Glial fibrillary acidic protein</td>
<td><em>Schwann cell marker</em>&lt;br&gt;- Non-myelinating</td>
</tr>
<tr>
<td>MAG Myelin-associated glycoprotein</td>
<td><em>Schwann cell marker</em>&lt;br&gt;- Pre-myelinating, where Schwann cells engulf axons destined to be myelinated</td>
</tr>
<tr>
<td>MBP Myelin basic protein</td>
<td><em>Schwann cell marker</em>&lt;br&gt;- Myelinating</td>
</tr>
<tr>
<td>NGF Nerve growth factor</td>
<td><em>Neurotrophin</em> &lt;br&gt; <em>trkA receptor</em></td>
</tr>
<tr>
<td>K14 Keratin 14</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Lineage tracing of epithelial cells</td>
</tr>
<tr>
<td>C/EBPδ,</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Quiescent</td>
</tr>
<tr>
<td>Bmi1</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Quiescent</td>
</tr>
<tr>
<td>SOX9</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Quiescent</td>
</tr>
<tr>
<td>ΔNp63α</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Quiescent, early activated,</td>
</tr>
<tr>
<td>ΔNp63β</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Activated, transient amplifying cells</td>
</tr>
<tr>
<td>ΔNp63γ</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Activated, transient amplifying cells</td>
</tr>
<tr>
<td>Wnt/β-catenin</td>
<td><em>Limbal stem cell marker</em>&lt;br&gt;- Activated, transient amplifying cells</td>
</tr>
<tr>
<td>β-iii tubulin</td>
<td><em>Axonal marker</em>&lt;br&gt;- Microtubule element of tubulin family found in neurons</td>
</tr>
<tr>
<td>Ki67</td>
<td><em>Cell proliferation marker</em></td>
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</tbody>
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**Table 3-1. Antibodies for future directions.** A description of antibodies that can be used for future investigations and what their indication is in the cornea.
Corneal sensory nerves contain several nerve-derived peptides that play a role in epithelial maintenance and healing in the cornea. The majority of corneal nerves contain either SP or CGRP, both of which were evaluated in the present thesis. Some nerves in the cornea also contain pituitary adenylate cyclase-activating peptide, galanin, vasoactive intestinal polypeptide, and met-enkephalin (Müller et al., 2003).

CGRP facilitates epithelial cell migration, and is involved in epithelial wound healing (Reid et al., 1993; Mikulec and Tanelian, 1996). SP stimulates corneal epithelial cell proliferation (Reid et al., 1993; Garcia-Hirschfeld, Lopez-Briones and Belmonte, 1994), upregulates corneal epithelial cell migration, in conjunction with insulin-growth factor 1 (IGF-1) (Nakamura, Nishida, et al., 1997; Chikama, Nakamura and Nishida, 1999) and increases the expression of tight-junction proteins in the corneal epithelium (Araki-Sasaki et al., 2000; Ko, Yanai and Nishida, 2009). SP is the most extensively studied neuropeptide in the literature, and has been shown to decrease in patients with corneal hypoesthesia (Stone and Kuwayama, 1985; Yamada et al., 2000). Capsaicin administration, which depletes SP and prevents the peptide from binding to its receptors, results in corneal changes consistent with NK including corneal opacity and corneal axonal loss (Fujita et al., 1984; Shimizu et al., 1987). Further, topical SP in conjunction with IGF-1 has been shown to heal persistent epithelial defects (PEDs) in animals (Murphy et al., 2001; Nagano et al., 2003) and patients (Brown et al., 1997; Chikama et al., 1998; Yamada et al., 2008). Presumably the presence of SP, and CGRP, in the neurotized cornea suggests the trophic influence of the nerve-derived peptides is restored, but this hypothesis needs to be confirmed with further studies.

SP upregulates tight-junction proteins, E-cadherin (Araki-Sasaki et al., 2000) and ZO-1 (Ko, Yanai and Nishida, 2009) in the corneal epithelium. SP is also implicated in facilitating the adhesion of epithelial cells to the underlying basement membrane and extracellular matrix through the upregulation of the aforementioned adhesion proteins as well as integrins. Further, trigeminal ganglions, containing SP (Lehtosalo, 1984), influence the expression of collagen VII on rabbit corneal epithelial cells in vitro (Baker et al., 1993), a key component of the hemidesmosome anchoring complex. Innervation and SP are
hence involved in formation of the hemidesmosome-anchoring complex. We hypothesize that corneal neurotization increases the expression of tight junction proteins, ZO-1 and E-cadherin, as well as of proteins associated with the anchoring complex, including ß-4 integrin (Future direction 1). Immunohistochemistry will be used to localize expression of these proteins (antibodies described in Table 3-1), as well as for semi-quantitation. Protein and mRNA semi-quantification can be performed using Western blot and Real Time -Polymerase Chain Reaction (RT-PCR) respectively. Capsaicin can be used to evaluate if the hypothesized upregulation of adhesion proteins in the neurotized cornea is reversed by prohibiting SP function, thus confirming the neuropeptide’s trophic influence on the reinnervated corneal epithelial integrity.

SP stimulates epithelial cell proliferation in vitro (Reid et al., 1993; Garcia-Hirschfeld et al., 1994), and wound healing in mouse models of diabetic keratopathy (Yang et al., 2014). In the hypo-innervated diabetic rabbit cornea, the epidermal growth factor (EGF) receptor does not phosphorylate, thus is not activated (Xu nd Yu, 2011). This phosphorylation is necessary in corneal epithelial cell turnover and wound healing which is mediated by the EGF-signalling pathway (Nakamura, Nishida, et al., 1997; Peterson et al., 2014; Rush et al., 2014). SP re-activates this signalling pathway, thereby promotes wound healing in diabetic mice (Yang et al., 2014). Thus, to determine whether the trophic influence of SP is restored in the denervated cornea treated with corneal neurotization, as we would expect, we will evaluate the presence and protein levels of phosphorylated EGF receptors (pEGFR) using immunohistochemistry and Western blot analysis respectively (antibody description in Table 3-1). We can confirm these results by evaluating changes in ocular surface health and pEGFR levels in the neurotized cornea whilst using capsaicin to block SP binding to its receptors. We hypothesize pEGFR levels will be restored in the neurotized cornea. This would suggest that SP, co-localized with the reinnervating axons in the treated denervated cornea, restores trophic support by influencing epithelial proliferation through the EGF signalling pathway.

In this thesis, our observations of the denervated corneal epithelium, including thinning and ulceration, suggest an impairment in epithelial adhesion and proliferation in our model
of NK. Our observed improvements in epithelial integrity of the denervated cornea treated with corneal neurotization suggest there is an improvement in these SP and CGRP-mediated molecular processes. Presence of nerve-derived peptides in the neurotized cornea suggests the trophic influence of these peptides may be restored. We hypothesize the pEGFR and adhesion protein levels will be restored in the neurotized cornea, and that this restoration and the improvement in ocular surface health observed in the neurotized cornea (Chapter 2), will be at least partially reversed by capsaicin administration.

FUTURE DIRECTION 3 – SCHWANN CELLS
In chapter 1.3 of this thesis, we briefly discussed the understudied Schwann cell (SC) presence in the cornea. The SCs are present in both the cornea and limbus (Figure 3-1). However, the function these SCs in the cornea and/or limbus is unknown, and no literature has been published regarding SC response to corneal denervation or hypoinnervation.

Figure 3-1 Schwann cells ensheath axons in the cornea and limbus. **Left.** Early cross-section of rat cornea and limbus (overview image), demonstrating βIII tubulin–positive axons (green) ensheathed by Necl-4 (white) and GFAP (red)–positive Schwann cells. Scale bars: 200 μm and 20 μm, respectively. **Right.** Cross-section of the rat cornea at limbal region (overview image), demonstrating a cross-section of single βIII tubulin–positive axon ensheathed by MAG–positive non-myelinating Schwann cells. Scale bars: 50μm and 2.5 μm in the, respectively.
In response to peripheral nerve injury SCs de differentiate and proliferate to participate in axonal regeneration (Stoll and Müller, 1999). Long-term denervation of tissues results in a reduced ability of these SCs to support nerve regeneration and a decline in SC gene expression (Fu and Gordon, 1997; You et al., 1997; Höke et al., 2002; Höke, 2006; Brushart et al., 2013). We hypothesize that corneal SC numbers will thus decrease in response to long-term corneal denervation. Investigating presence and localization of SCs can be performed with immunohistochemical staining for SC antibodies (Necl4, GFAP, MAG; Table 3-1) in the normally innervated cornea and denervated cornea of our NK rat model, and SC levels can be confirmed with Western blot analysis both corneal groups.

Figure 3-2 Schwann cells are located in close proximity to Sox9 positive cells. Extended depth focus immunofluorescent image of limbal area of whole mount rat cornea. Sox9-positive nuclei, possibly LSC (red), are in close vicinity to Necl-4–positive Schwann
cells (green) ensheathing axons in the limbus. The border between the limbus and cornea is annotated by a dashed line. Scale bars: 50 μm.

In our preliminary staining of the whole-mount limbal epithelium, we observed Sox9 positive cells, possibly indicating LSCs, located in close proximity to axon-ensheathing Necl4 positive SCs in the limbus (Figure 3-2). Although the relationship between SCs and LSCs has not yet been investigated, the close proximity of these cells to each other indicate they may influence one another. In fact, SCs in the bone marrow have been found to support homeostasis of hematopoietic stem cells (Yamazaki et al., 2011; Yamazaki and Nakauchi, 2014) and release neurotrophins, including NGF, brain derived neurotrophic factor (BDNF), and others, in response to axonal injury (Meier et al., 1999; Fontana et al., 2012; Brushart et al., 2013). Neurotrophins including NGF, neurotrophin-3 and 4/5, and BDNF, and their receptors are present in the cornea. Further NGF’s trkA receptors are specifically located on the limbal epithelium, where the LSCs are found. This suggests that if SCs are a source of NGF and other neurotrophins, these cells may potentiate LSC function through the release of the neurotrophins. Perhaps corneal epithelial wounding results in intraepithelial axonal injury, stimulating the release of NGF from SCs to act on its receptors on LSCs (Figure 3-3). This hypothesis was formed by applying knowledge about SC function(s) in other tissues in the context of the cornea. Therefore, many techniques and studies need to be performed to test this hypothesis and to elucidate the function of SCs and the underlying mechanism(s) in the cornea specifically. In our current rat model of NK, immunohistochemical staining and Western blot can be used to first evaluate neurotrophin (NGF) and neurotrophin receptor (trkA) presence in the normally innervated cornea, and subsequently in the denervated cornea (Table 3-1). However, even if the SC phenotype changes, for example these SCs de-differentiate, or SCs are lost in response to corneal denervation, the changes we expect to see in neurotrophin and neurotrophin receptor levels could not be exclusively attributed to loss of innervation nor loss of normal SC phenotype. Cell-culture experiments can be used to evaluate if isolated corneal SCs, on pre-conditioned media, release growth factors including NGF.
**Figure 3-3 Proposed mechanism of action of corneal SCs in response to axonal injury.** Schematic diagram – we propose that following axonal injury SCs release NGF which acts on its trkA receptors in the limbal epithelium to stimulate LSC proliferation and differentiation.

Several transgenic mice exist and thus, translating our rat NK and corneal neurotization models into mice offers greater research potential than our current rat model. The relationship between NGF’s receptor trkA and innervation has previously been investigated in a transgenic trkA knock-out mouse model, in which trkA (-/-) mice had reduced corneal sensation and nerve terminals (de Castro et al., 1998). Further, trkA is co-localized with several LSC-associated markers (Touhami et al., 2002; Qi et al., 2008). Tamoxifen application in the trkA\textsuperscript{CreERT2/+};R26-LSL-TdT;R26-LSL-DTA mouse will induce apoptosis of trkA positive cells exclusively in the cornea by the activation of the diphtheria toxin. Immunolabelling of NGF, β-III tubulin, SC and LSC-associated markers
in the cornea of this mouse model may help elucidate the proposed mechanism in Figure 3-3.

Stereotactic ablation of the ophthalmomaxillary nerve to achieve corneal denervation has previously been performed in mice (Ferrari et al., 2011) and can be used in transgenic mouse models to study the relationship between corneal SCs and innervation. In the Sox2\textsuperscript{CreERT2/+,R26-LSL-TdT} mouse, topical tamoxifen application induces TdTomato expression on Sox2-positive cells, SCs. Co-localization of TdTomato and other SC markers (Table 3-1) in the transgenic mouse cornea will confirm this model as suitable for use in further corneal SC investigations. In vivo fluorescent confocal microscopy can then be used, in this mouse model, to track what happens to SCs in the days and weeks following corneal denervation. Further, we can use transgenic mouse models to selectively ablate SCs in the mouse cornea, without damaging axons. Topical tamoxifen application in the Sox2\textsuperscript{CreERT2/+,R26-LSL-TdT;R26-LSL-DTA} mouse will induce apoptosis of SC (Sox-2 positive) cells by activation of the diphtheria toxin. This ablation will be confirmed by disappearance of TdTomato labelled cells and of SC marker expression. This model can be used to observe possible changes to corneal innervation, through β-III tubulin immunostaining, and observations of changes in ocular surface health and healing. The effect of SC ablation on the presence of NGF, other neurotrophins and their receptors can also be assessed with immunostaining (Table 3-1).

Once SC response to corneal denervation is established, we can investigate SC presence and function in the denervated cornea after treatment with corneal neurotization. Using immunohistochemical staining in our current rat model, we can investigate whether there are SCs in the re-innervated cornea, where they are expressed and what their function is, if any. While the majority of native corneal nerves are unmyelinated, the nerve autografts used in corneal neurotization contain a diverse population of both myelinated and unmyelinated nerves. Thus, in our established rat model, immunostaining with a myelinating SC marker, such as MBP, and non-myelinating SC marker, such as GFAP, can be used to confirm whether the reinnervating axons from the autografts are myelinated or unmyelinated (Table 3-1).
Development of a mouse model is necessary for the following proposed investigations. The experiments in transgenic mouse models described above can be repeated in the neurotized mouse cornea. Catapano et al (2018) demonstrated that only a small proportion of the axons regenerating through the autografts reinnervated the denervated cornea in our rat model of NK. In vitro, long-term denervated SCs retain the capacity to re-activate to support axons (Gordon et al., 2019). Thus, it is possible that the regenerating axons are guided to the cornea by remaining non-myelinating SCs native to the denervated cornea which are re-activated by close proximity to the nerve grafts. Alternatively, it is possible the SCs of the nerve autograft may in some way migrate into the cornea, and perhaps aid corneal reinnervation. By using transgenic mouse models, we can investigate whether nerve isograft derived fluorescently-labelled SCs migrate to the cornea to support axon regeneration and corneal reinnervation, or if the native SC remain or re-activate to proliferate and support re-innervation following corneal neurotization. The aforementioned Sox2<sup>CreERT2/+</sup>;R26-LSL-TdT mouse can be used to selectively induce TdTomato expression of the SCs in the harvested sural and common peroneal isografts in the corneal neurotization procedure. These isografts can then be used to reinnervate the cornea of a genetically identical mouse lacking SC activation by tamoxifen and, at the end-point, harvested corneas can be immunolabelled for SC markers (Table 3-1). Co-localization of TdTomato expression with SC markers would suggest the SC in the re-innervated cornea are derived from the isograft. Conversely, absent TdTomato expression or absent co-localization would suggest the native corneal SCs remain in the denervated cornea.

**FUTURE DIRECTION 4 – LIMBAL STEM CELLS**

There is an ill-defined relationship between limbal epithelial stem cells (LSCs) and corneal/limbal sensory innervation, but LSCs and corneal innervation are critical for corneal epithelial wound healing. Because of differences in symptoms between LSC deficiency and NK patients, the absence of corneal innervation likely does not result in a reduced LSC quantity. However, perhaps innervation influences LSC differentiation and proliferation in response to epithelial cell turnover and wound healing. Although a
definitive LSC marker does not exist, several have been suggested in the literature and it is suggested that LSC marker expression changes in response to LSC activation. K14 is an accepted LSC marker which has been used for epithelial cell lineage tracing in mouse models (Figueira et al., 2007; Amitai-Lange et al., 2015; Nasser et al., 2018). Quiescent LSCs express C/EBPδ, Bmi1, SOX9, ΔNp63α, and activated LSCs/transient amplifying cells express ΔNp63β, ΔNp63γ and Wnt/β-catenin (Di Iorio et al., 2005; Barbaro et al., 2007; Menzel-Severing et al., 2018). We hypothesize that in the absence of innervation, LSCs will remain in a quiescent state and fail to activate to become transient amplifying cells.

We performed a preliminary microarray analysis of the RNA of the limbal areas dissected from the normally innervated and denervated rat corneas 2 days after corneal epithelial wounding. There were several significant differences in gene expression between the two groups (Figure 3-4). However, the limitation of this method is that we were not able to specifically isolate the LSCs. Single cell-sequencing would allow us to evaluate specific differences in LSC gene expression between the normally innervated and denervated rat corneas and target these differences in gene expression for futures research. We are currently optimizing the techniques used to isolate LSCs in culture, to be used for single-cell sequencing analysis.
Figure 3-4 Preliminary microarray analysis. A. Microarray analysis of limbus of normally innervated and 7-day denervated rat corneas 2 days after corneal epithelial injury. B. Quantification demonstrates significant changes (downregulation in green and upregulation in red) in gene expression between the denervated and normally innervated groups.

In our existing NK rat model, we will use immunohistochemical staining to evaluate differences in LSC marker (Table 3-1) expression with and without epithelial wounding in the normally innervated and denervated corneas. Topical BrdU application after epithelial wounding can identify LSCs as slow-cycling label-retaining cells (Figueira et al., 2007). Ki67, a proliferation marker (Table 3-1), can be stained for in conjunction with the LSC markers to specifically look at the proliferation of these cells. If a difference in marker expression is found, this can be repeated in the neurotized cornea to evaluate whether the re-innervating nerves influence the LSC activation in the same way as the native corneal sensory nerves.

In the Sox9CreERT2/+;R26-LSL-TdT mouse, tamoxifen application induces TdTomato expression of Sox9-positive cells. In the normally innervated cornea, the LSC phenotype of these cells will be confirmed by immunolabelling with other LSC markers (Table 3-1) and assessing co-localization of marker expression with TdTomato expression. Corneal denervation in this model can be used to evaluate changes in LSC expression in the absence of innervation. The R26-confetti/K14-Cre mouse model allows lineage tracing of the corneal epithelium (Nasser et al., 2018). Topical application of tamoxifen in these mouse corneas induces K14-positive cells (LSCs) to express a fluorescent protein (green, red, cyan, yellow) and allows for lineage tracing of terminally differentiated epithelial cells (Amitai-Lange et al., 2015; Nasser et al., 2018) (Figure 3-5). Thus, reduced or absent K14 lineage tracing would be observed if LSC function is impaired. Corneal denervation in this mouse model can be used to test the hypothesis that LSC function is impaired in the absence of innervation.
The ability to cross transgenic mice (R26-confetti/K14-Cre and Sox2<sup>CreERT2/+</sup>;R26-LSL-TdT;R26-LSL-DTA) may allow for investigation of LSC response to SC-induced death in the normally innervated cornea. This would further confirm or deny SC influence on LSCs, as discussed in “future direction 3,” and help elucidate whether the hypothesized impairment in LSC function is due to loss of corneal innervation or SCs. Lastly, the above LSC investigations using mouse models can be repeated in the neurotized mouse cornea to assess the effect of re-innervation on the denervated cornea’s LSCs.

**Figure 3-5 Transgenic confetti mouse.** Lineage tracing from a R26-confetti/K14-Cre mouse. K15 positive cells induced to express Green (GFP) Red (RFP) Cyan (CFP) and Yellow (YFP) fluorescent proteins as they differentiate from the LSCs in the limbus (indicated by dotted line) to terminate as epithelial cells in the central cornea. (Modified from Stem Cells, Amitai-Lange et al, Lineage tracing of stem and progenitor cells of the murine corneal epithelium pages 230-239, copyright (2014) with permission from Elsevier: License # 4595700226918)

The use of transgenic mouse models and *in vivo* confocal microscopy allows for countless manipulations to corneal innervation and cells to be studied in live animals and in real-time. Because of its transparency, the cornea is an ideal tissue in which to study the relationship between innervation, stem cells and SCs, and could have implications in a broader context beyond preservation of corneal health in NK.
FUTURE DIRECTION 5 – TOPICAL AGENTS

In the majority of patients, the corneal neurotization procedure is effective in treating NK. However, some patients do not benefit from the procedure (Catapano et al., 2019). Identifying an underlying mechanism involved in corneal epithelial maintenance or healing that is not restored following corneal reinnervation may help elucidate a target for future interventions. A topical agent could then perhaps be used to supplement the current neurotization surgical procedure to improve outcomes. Below I outline some possible topical agents, based on our current knowledge, and propose experiments to test their efficacy.

Corneal neurotization treats the underlying condition of NK by restoring innervation in the affected cornea. In the denervated rat cornea, we have previously shown corneal neurotization improves healing over time (Catapano et al. 2018). It is likely that just as numerous factors contribute to the impairment in healing in NK, several factors may contribute to the observed improvement in healing following corneal neurotization. Although not yet confirmed, we hypothesize the improvement in healing observed (Catapano et al. 2018) is due to the restoration of nerve-derived peptides, such as SP and CGRP. As previously discussed in Chapter 1.3 and 1.4 of this thesis, in the normally innervated cornea these peptides provide trophic support to the cornea by promoting adhesion (Araki-Sasaki et al., 2000; Ko, Yanai and Nishida, 2009), proliferation (Reid et al., 1993; Garcia-Hirschfeld, Lopez-Briones and Belmonte, 1994), and migration (Nakamura, Nishida, et al., 1997; Chikama, Nakamura and Nishida, 1999) of epithelial cells. Previous groups have demonstrated that topical agents containing these nerve-derived peptides are most effective in healing epithelial wounds when combined with other trophic factors, such as SP with IGF-1 or CGRP with SP (Nishida et al. 1996; Nakamura et al., 1998; Chikama et al., 1999; Ko et al. 2013). Although, in Chapter 2 of this thesis we have shown that corneal neurotization restores the presence of the nerve-derived peptides SP and CGRP, the trophic activity of these peptides in the neurotized denervated cornea needs to be confirmed. To test whether neurotization restores trophic support, we will repeat the previously published corneal healing experiment (Catapano et al. 2018) in the denervated + neurotized rat cornea with topically applied capsaicin. This
Topical agent will ablate the effect of SP, one of the nerve-derived mediators that provides the cornea with trophic support. An observed impairment in healing in this model, would confirm the previously documented improvement (Catapano et al., 2018) was, at least in part, due to the restoration of SP. Further, perhaps topical agents containing SP and/or CGRP will facilitate corneal neurotization’s improvement in corneal epithelial healing and health in the denervated cornea.

Topical agents containing NGF promote corneal epithelial cell differentiation, proliferation and migration in a corneal injury model in hens (Blanco-Mezquita et al., 2013) and rodents (Lambiase et al., 2000). In patients, topical NGF effectively heals corneal PEDs of a variety of etiologies (Bonini et al., 2000a, 2000b; Tan, Bryars and Moore, 2006; Lambiase, Sacchetti and Bonini, 2012; Sacchetti, Lambiase and Ph, 2017), despite the lack of knowledge of the source and mechanism of action of the neurotrophin in the normal healthy cornea (Bonini et al., 2000a; Lambiase et al., 2000; Vesaluoma et al., 2000; Müller et al., 2003). However, success in NGF treatment is primarily documented in cases of corneal hypoinnervation, not complete denervation (Lambiase, Se, et al., 1998; Bonini et al., 2000; Tan et al., 2006; Lambiase et al., 2012; Sacchetti et al., 2017). Because corneal neurotization treats the underlying cause of NK, it is likely that NGF is not as effective in improving corneal epithelial healing and health of the completely denervated cornea. However, it is possible that in combination with the procedure, topical NGF would advance the previously observed improvement.

As discussed above, SCs that are present and ensheath axons in the cornea and limbus may be a source of growth factors, including the neurotrophin NGF. In response to peripheral nerve axonal injury, SCs are known to release growth factors such as NGF (Meier et al., 1999; Fontana et al., 2012; Brushart et al., 2013), as well as support axon and digit-tip regeneration, epithelial wound healing and stem cell function (Jessen and Mirsky, 2016; Carr and Johnston 2017). Schwann cell conditioned media (SCcm) consists not only of NGF (Brushart et al., 2013), but also of other factors SCs release such as NT-3, and PDGF (Meier et al., 1999; Brushart et al., 2013). Thus, if in patients, topical
application of NGF is effective in healing PEDs, presumably SCcm would be at least as effective.

FK506 (tacrolimus) is commonly used as an immunosuppressant agent in corneal applications such as preventing rejection of corneal grafts (Wu et al., 2019) and LSC transplants (Ballios et al., 2018). Our group has shown local FK506 enhances peripheral nerve regeneration (Tajdaran et al., 2015, 2016b) by promoting proliferation of SCs (Tajdaran et al., 2016a). However, the nerve regeneration capabilities of this drug have not been studied in the cornea. This drug is FDA and Health Canada-approved and is available as a topical ophthalmologic agent. Thus, as with topical applications of SP, CGRP, NGF, and SCcm, this drug may aid the advancement of epithelial healing and health in the neurotized denervated cornea.

We have previously published an experiment in which we observed corneal neurotization improves corneal healing over time as compared to the denervated cornea that received no treatment (Catapano et al., 2018). In future experiments, we will use the same experiment paradigm and experimental groups to test the hypotheses that in the denervated cornea 1) corneal neurotization is more effective in healing epithelial defects than the aforementioned topical agents alone, and 2) corneal neurotization is more effective in healing corneal epithelial defects in combination with the topical agents than alone. To allow for comparisons to our previously published work, the groups used will be as they were previously: (A) normally innervated corneas (control), (B) denervated corneas (negative control), and (C) denervated + neurotized corneas. The topical agents used will be i) SP + IGF-1 dissolved in Hanks Balanced Salt Solution (HBSS), ii) CGRP + SP in HBSS, iii) NGF in HBSS, iv) SCcm, v) FK506 vi) HBSS (negative control) and vii) SCpm (SC pre-conditioned media, negative control for SCcm).

Prior to use of topical agents in vivo, the activity of the agents will be tested in vitro. The concentrations used will be based on previously published literature (Nishida et al. 1996; Nakamura et al., 1998; Chikama et al., 1999; Lambiase et al. 2000; Bonini et al., 2000a, 2000b; Tan, Bryars and Moore, 2006; Lambiase, Sacchetti and Bonini, 2012; Ko et al.
2013; Sacchetti, Lambiase and Ph, 2017). SP and CGRP have been shown to promote the stratification of corneal epithelial cells (Ko et al., 2013), thus the efficacy of i), ii) and vi) will be tested in cultures of human corneal epithelial cells. FK506, NGF, and SCcm all promote neurite outgrowth, and thus the efficacy of iii) iv), v), vi) and vii) will be tested on DRG cultures. Once concentrations are confirmed to ensure maximum effect in vitro, the topical agents will be used to promote healing in the three groups in vivo. Further, to ensure the topical agents are acting on-target, they can be applied with receptor-antagonists.

The protective tarsorrhaphy will be removed, four weeks after the initial denervation, and the corneal epithelium will be carefully debrided using an Algerbrush II (Alger Company, Inc., Lago Vista, TX, USA). Fluorescein (DioFluor Strips, Innova Medical Ophthalmics, Inc) will be applied to the cornea and the staining imaged digitally (Nikon D 5100; Nikon) under a ultraviolet lamp each day prior to application of topical drops applied to the denervated cornea. Topical drops will be applied 4X per day between 8 AM and 8 PM. Imaging will be performed using a standardized frame to keep the camera a fixed distance from the corneal surface. The area of epithelial injury will be measured using ImageJ. The percent of the corneal area healed over time will be calculated by standardizing the de-epithelialized area at each timepoint to the initial wound size. The mean (± SD) percent of corneal area healed in each group at each time point will be calculated and compared using unpaired t-tests. In contrast to the previously published experiment, we will increase the time of observation to one week. Though, with the addition of topical agents, we hypothesize healing to improve within the four days it took for an epithelial wound to heal in the denervated + neurotized cornea, we expect it will take longer for a wound to heal, if at all, with applications of topical agents on the completely denervated cornea.

The experiments performed in Chapter 2 in this thesis, evaluating corneal epithelial and stromal thicknesses, and evaluating corneal epithelial ulceration, scarring and perforation, can similarly be repeated with the aforementioned topical agents and groups.
Of all the previously discussed topical agents, NGF is the treatment that has the largest documented success in healing epithelial defects in patients. Even so, the documented successes have been primarily in patients with corneal hypoesthesia, decreased sensation, rather than complete anesthesia, absent sensation (Lambiase et al., 1998; Bonini et al., 2000; Tan et al., 2006; Lambiase et al., 2012; Sacchetti et al., 2017). It is therefore reasonable to hypothesize the other proposed topical agents would similarly be more efficacious in cases of hypoinnervation. In our current model of NK, to achieve complete denervation of the cornea for four weeks, we perform the ophthalmomaxillary ablation twice. In the absence of the second ablation we have observed the early stages of corneal reinnervation (Catapano et al., 2018) that resembles corneal hypoinnervation. Thus, in the future we will re-evaluate the effect of topical SCcm and NGF application on healing in the completely denervated (two ablations) and hypoinnervated (one ablation) cornea over a longer period of time. This can also be done using corneal ablation in healthy mice and mouse models of hypoinnervation such as herpetic keratitis in which NGF topical treatment was effective in healing epithelial injury (Lambiase et al., 2008). Repeating the healing experiment with the addition of topical agents in a corneal hypoinnervation model (group D) might be extremely informative and applicable for patients.

In cases of corneal hypoesthesia due to metabolic diseases, such as in diabetic or herpetic keratopathy, corneal neurotization may not be a suitable option because of a lack of viable donor nerves for coaptation of the nerve autograft. In cases where hypoesthetic NK develops as a result of lamellar flap surgery, corneal abrasions, corneal transplants, and LASIK, for example, less severe symptoms may not necessitate the invasive, relative to topical drops, neurotization procedure. If corneal SCs are in fact the source of NGF and other growth factors or neurotrophins, SCcm could be more effective in treating PEDs than NGF alone. This is a similar, but more specific approach to treatment of NK with autologous serum (Matsumoto et al., 2004). Contrary to the autologous serum, the corneal SCcm would solely consist of the growth factors specifically released by SCs in the cornea, and no other factors or molecules beyond what is normally present in the healthy cornea. Further, FK506 is a promising drug for
topical use to enhance corneal nerve regeneration in hypoinnervated corneas because of its effectiveness in enhancing nerve regeneration in other tissues, and its safety for ophthalmic use in patients.

COLLABORATIONS FOR FUTURE DIRECTIONS
Our laboratory has developed the only animal model of corneal neurotization in rats, as well as an animal model of NK with the longest-documented period of corneal denervation (4 weeks). We have, in this thesis, established protocols for immunohistochemistry and Western blot analysis of rat corneas and have experience with these techniques in peripheral nerve analysis. Many of the methods proposed for future directions, such as use of transgenic mouse models, necessitates collaboration with experts in other fields. Collaboration with the Kaplan-Miller laboratory (The Hospital for Sick Children, Toronto) will greatly benefit our investigations into corneal SCs. Expertise in the area of LSCs will be provided through collaboration with the Shalom-Feuerstein laboratory (Technion, Israel). Collaboration with both of these laboratories will be instrumental in understanding the underlying mechanisms involved in corneal epithelial maintenance in the normally innervated, denervated and neurotized corneas and how this relates to SCs and LSCs.

3.3 – Concluding remarks
This thesis demonstrates corneal neurotization is effective in maintaining corneal epithelial integrity and health in the denervated rat cornea of our NK model. Corneal neurotization is more effective than topical applications of NGF in treating epithelial injury in the completely denervated cornea. Presence of nerve-derived peptides, SP and CGRP, suggests a possible mechanism by which epithelial integrity is maintained, but further studies need to be done to support this claim.

Despite the procedure’s demonstrated effectiveness in maintaining and healing the corneal epithelium in both our NK rat model (Chapter 2; Catapano et al., 2018) and patients (Catapano et al., 2019), about 15% of patients fail to reach levels of protective
corneal sensation post-operatively (Catapano et al., 2019). Additionally, once NK symptoms progress to affect corneal layers deeper than the superficial epithelium, re-innervating the cornea will not reverse the existing scarring or opacification (Catapano et al., 2019) and these patients may still require corneal transplants/keratoplasties. In most clinical cases patients are seen in late-stage NK, already presenting with moderate to severe complications. The most direct application of this model and this study is thus for patients with early stage NK, without severe ulceration or scarring. This emphasizes the importance of identifying and treating these patients as early as possible.

This thesis work and our outlined future directions (Chapter 3.2) enhances and will add to the understanding of 1) the relationship between innervation, SCs and LSCs, 2) the molecular basis of NK, and 3) the mechanisms underlying the improvement in ocular surface health following neurotization of the denervated cornea. The ultimate goal of research into corneal innervation is to benefit the NK patients that do not currently benefit from the corneal neurotization procedure. We believe our investigations into this area will benefit patients that suffer from other ocular surface diseases, and perhaps have implications in the broader context of peripheral nerves, SCs and stem cells.
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