CORNEAL REINNERVATION TO TREAT NEUROTROPHIC KERATOPATHY

by

Joseph Catapano

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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2016

Abstract

Patients lacking corneal sensation develop neurotrophic keratopathy (NK). NK is characterized by recurrent breakdown of the corneal epithelium, resulting in scarring of the cornea and permanent vision loss. Even with optimal ophthalmic management, corneal epithelial breakdown and vision loss in patients with advanced NK is inevitable. Conventional treatments protect the cornea from injury but fail to prevent vision loss as they do not address the underlying lack of corneal innervation. Success in treating other nerve palsies surgically with nerve grafts and transfers provides a strong rationale for investigating surgical reinnervation of the cornea in patients with NK. We demonstrate in a prospective clinical study that a novel surgical procedure to reinnervate the cornea improves corneal sensation and ocular surface health in patients with NK. Furthermore, we document reinnervation of the cornea using immunohistochemistry, magnetoencephalography and in vivo confocal microscopy of the cornea. We developed a novel rat model of NK and corneal reinnervation with nerve grafts and transfers to further investigate how foreign axons influence corneal epithelial healing. In this model, we demonstrated that corneal reinnervation improves corneal healing after injury, suggesting that the donor nerves that reinnervate the cornea contain neuromediators necessary to promote corneal epithelial healing.
There are several challenges limiting axon regeneration and recovery when using nerve grafts and transfers. In the final chapter, investigation of retrograde neuronal death after neonatal peripheral nerve injury demonstrated that N-acetyl cysteine decreases motor neuron death but fails to prevent sensory neuron death after injury. The promising clinical results and animal studies presented as part of this thesis suggest reinnervation of the cornea with nerve grafts and transfers results in corneal reinnervation, restoring corneal sensation and nerve-derived trophic support, which in turn, improves corneal epithelial maintenance and repair after corneal injury in the context of NK. With this work, we have introduced an animal model that will allow investigation of the mechanisms by which corneal reinnervation influences the corneal epithelium and vision.
Acknowledgments

I owe my sincerest gratitude to my supervisor, Dr. Gregory Borschel, for his guidance, mentorship and encouragement. Thank you for sharing your wisdom, challenging me, and teaching me the skills necessary to succeed and continue research in the future.

I would also like to thank my thesis committee members, Drs David Kaplan, Derek van der Kooy, and Tessa Gordon. Your guidance and experience were crucial to my education, and the lessons I learned from you will guide my career.

Thank you to my colleagues in the Borschel Laboratory who supported me throughout my PhD. Jennifer Zhang, Michael Willand, Kasra Tajdaran, Michael Hendry: your support and expertise have improved the quality of my work and have prepared me to be a better researcher and clinician. Thank you as well to the undergraduate students we had the privilege of working with. Kira, Cameron, David, Mark and Katelyn; your contributions were invaluable.

I would also like to thank Emily Ho, Drs Asim Ali, Simon Fung, Douglas Cheyne and Cecilia Jobst. Your expertise and support were crucial to the clinical investigation of corneal neurotization. I would like to acknowledge the support and guidance of Drs Christopher Forrest, Mitchel Brown, and the Department of Surgery. Without your support, I would not have been able to pursue this opportunity.

Thank you to my family, who have always supported and encouraged me. Mom and Dad, I am blessed to have parents as caring as you, who always stood by me and instilled in me the importance of hard work and perseverance. To my brothers Daniel and Michael, your advice helps put things in perspective and you are two of the best friends I could ever hope for.

I would like to especially thank my wife, Ines. Thank you for supporting me every step of the way, and being there every day to remind me that health, happiness and family are most important. You are a truly special person and you encourage me to be the best version of myself. And finally, to my daughter Matea: your arrival has enriched the lives of everyone around you. I look forward to all the exciting adventures we will share in the future.
I would also like to acknowledge the following programs which supported my research: Canadian Institute of Health Research (CIHR) Frederick Banting and Charles Best Graduate Scholarship, University of Toronto School of Graduate Studies (Hilda and William Courtney Clayton Pediatric Research Fund, Peterborough K.M. Hunter Graduate Studentship), Faculty of Medicine (Edward Christie Stevens Fellowship and Joseph M. West Memorial Fund), Department of Surgery (Alumni Fellowship), Division of Plastic and Reconstructive Surgery (Mentor Medical Systems Canada Graduate Scholarship), Hospital for Sick Children (Research Training Award), Plastic Surgery Foundation (PSF), American Society of Peripheral Nerve Surgery (ASPN), and Physician Services Incorporated (PSI)
Contributions

Joseph Catapano (author) solely prepared this thesis. All aspects of this work were completed in whole or in part by the author. The following contributions by other individuals are formally acknowledged:

Dr. Gregory Borschel (Primary Supervisor and Thesis Committee Member) – mentorship; laboratory resources; planning and execution of experiments; manuscript and thesis preparation

Dr. Tessa Gordon – planning and execution of experiments; interpretation of results, manuscript preparation and thesis preparation

Dr. David Kaplan (Thesis Committee Member) – mentorship; guidance and interpretation of results; thesis preparation

Dr. David van der Kooy (Thesis Committee Member) – mentorship; guidance and interpretation of results; thesis preparation

Dr. Jennifer J. Zhang – maintenance of the thy1-GFP+ colony; laboratory manager

Dr. Asim Ali and Dr. Ronald M. Zuker – performed corneal neurotization clinically in conjunction with Dr. Borschel; manuscript review for Chapter 2

Dr. Simon SM Fung – data interpretation; manuscript preparation for Chapter 2

Dr. Douglas Cheyne and Cecilia Jobst – performance of magnetoencephalography (MEG); analysis of MEG data and assistance with MEG data interpretation for Chapter 2

Dr. William Halliday – interpretation of histology data included in Chapter 2

Emily Ho – guidance of clinical experiments; navigation of the Research Ethics Board (REB)

Kira Antonyshyn – assistance with the analysis of corneal nerve density for Chapter 4

Cameron Chiang and David Scholl – assistance with tissue analysis Chapter 5

Katelyn Chan – assistance with developing the protocol for nerve density analysis in Chapter 4
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>BSCVA</td>
<td>Best spectacle corrected visual acuity</td>
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<td>CCS</td>
<td>Central corneal sensation</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
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<tr>
<td>CNFG</td>
<td>Cross-face nerve graft</td>
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<td>CN</td>
<td>Corneal neurotization</td>
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<td>CN V</td>
<td>Trigeminal nerve</td>
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<td>Common peroneal</td>
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<td>Dorsal root ganglia</td>
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<td>EGF</td>
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<td>EGFR</td>
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<td>ENT</td>
<td>Epithelial nerve terminals</td>
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<td>FG</td>
<td>Fluorogold</td>
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<td>GDNF</td>
<td>Glial-cell derived neurotrophic factor</td>
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<td>IVCCM</td>
<td>In-vivo corneal confocal microscopy</td>
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<td>ION</td>
<td>Infraorbital nerve</td>
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<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
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<tr>
<td>LP</td>
<td>Limbic plexus</td>
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<tr>
<td>MEG</td>
<td>Magnetoencephalography</td>
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<tr>
<td>NGF</td>
<td>Nerve-growth factor</td>
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<tr>
<td>NK</td>
<td>Neurotrophic keratopathy</td>
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<td>NT-3</td>
<td>Neurotrophin-3</td>
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<tr>
<td>PCS</td>
<td>Peripheral corneal sensation</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>RG</td>
<td>Retrograde</td>
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<tr>
<td>SBP</td>
<td>Sub-basal nerve fibers</td>
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<tr>
<td>SC</td>
<td>Schwann cell</td>
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<tr>
<td>SEN</td>
<td>Sub-epithelial nerve fibers</td>
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<td>SP</td>
<td>Substance P</td>
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<tr>
<td>SN</td>
<td>Stromal nerve trunks</td>
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<tr>
<td>TG</td>
<td>Trigeminal ganglion</td>
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<tr>
<td>V1</td>
<td>Ophthalmic branch of the trigeminal nerve</td>
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<tr>
<td>V2</td>
<td>Maxillary branch of the trigeminal nerve</td>
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<tr>
<td>V3</td>
<td>Mandibular branch of the trigeminal nerve</td>
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<td>WEST D</td>
<td>Semmes weinstein enhanced sensory test-D</td>
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Chapter 1  Introduction and Literature Review
1.1 Preamble

The cornea is the window through which we see the world. Tissue transparency is unique to the structures of the eye and corneal transparency is dependent on the tightly regulated organization of the cellular and extracellular components of the corneal matrix (Dawson et al. 2011). While the cornea is avascular in order to maintain tissue transparency, it is one of the most densely innervated structures in the body (Rozsa & Beuerman 1982; Guthoff et al. 2005; Beuerman & Schimmelpfennig 1980). Corneal sensation is a critical component of the corneal blink reflex that mechanically protects the eye from injury and the corneal nerves are a source of trophic mediators necessary for the maintenance of corneal clarity and healing after injury (Müller et al. 2003). Absent or impaired corneal innervation causes neurotrophic keratopathy (NK), a degenerative corneal disease characterized by occult corneal injury and progressive scarring (Lambley et al. 2014; Ramaesh et al. 2007; Rosenberg 1984).

Current ophthalmic management includes the use of lubricants and protective lenses to prevent corneal injury and biologic and anti-inflammatory agents to inhibit corneal neovascularization. Even with optimal ophthalmic management, patients require life-long treatment and many develop permanent vision loss (Lambley et al. 2014; Ramaesh et al. 2007). Current treatments are intended to manage the complications of neurotrophic keratopathy but they fail to address the underlying pathophysiology of absent or impaired corneal innervation. Corneal reinnervation has been attempted in the past by either surgically repairing the ophthalmic nerve (Samii 1981) which innervates the cornea, or by reinnervating the cornea using donor nerve transfers (i.e. corneal neurotization) (Terzis et al. 2009). These techniques however, have not been widely adopted to treat patients because of the excessive morbidity of each procedure, requiring either an osteoplastic frontal craniotomy or bicoronal incision. At the Hospital for Sick Children, a new less invasive technique of corneal neurotization was developed to reinnervate the cornea using two small supraorbital incisions and nerve grafts (Bains et al. 2015; Elbaz et al. 2014). Placement of nerve grafts and transfers is the established surgical technique to reconstruct motor deficits and reanimate the face in patients with facial paralysis from cranial nerve (CN) VII palsy (Terzis & Konofaos 2008; Lee et al. 2008), providing a strong rationale for their use in reconstructing sensory deficits of the cornea. Further investigation is required to document corneal reinnervation after neurotization and to determine whether donor nerves also restore the cornea with neuromediators that are necessary for ocular surface health and healing after injury.
1.2 Thesis Organization

This thesis is organized in a “multiple paper format”, using unaltered peer-reviewed content and unpublished data where indicated. This structure will provide readers with a better understanding of how the clinical studies provided the rationale for the laboratory work, and how this compliments and furthers our understanding of how corneal neurotization influences corneal health in patients with neurotrophic keratopathy (NK). Chapter 1 introduces corneal physiology in the context of NK as well as some of the present challenges limiting axon regeneration after nerve repair. The final section provides an outline of the research aims investigated in this thesis. Chapter 2 presents original research investigating the outcomes of corneal neurotization in a consecutive cohort of patients treated at The Hospital for Sick Children. The chapter has been reviewed by co-authors and reformatted from a paper presently in submission. Chapters 3 and 4 present original research developing a rat model of NK and corneal neurotization to further investigate how corneal neurotization influences maintenance and repair of the cornea. Chapter 5 has been reformatted from work published in the journal *Plastic and Reconstructive Surgery* (Catapano et al., 2017). This work investigates a pharmacologic means of reducing retrograde neuronal death after nerve injury, which is a challenge in optimizing outcomes after nerve transfers such as those used for corneal neurotization. Each chapter separately discusses the results and future directions in the context of existing literature. This is complimented by Chapter 6, which summarizes significant results and outlines ongoing and future directions.
1.3 Anatomy and Physiology of the Cornea

1.3.1 Introduction

Key aspects of the cornea and corneal nerve anatomy and physiology are summarized, including epithelial wound healing under normal circumstances.

1.3.2 Corneal Anatomy

The cornea is the interface between the ocular contents and the external environment. In addition to providing a barrier to infection and injury, the maintenance of corneal clarity and shape is a crucial aspect of vision (Figure 1.1A). Several factors contribute to corneal clarity. These include its thickness (540 to 700 µm), a thin layer of squamous epithelium, and a tightly regulated lattice-like arrangement of collagen fibrils (Hassell & Birk 2010). The cornea has 5 distinct layers (Figure 1.1B):

i. Corneal epithelium: 4 to 6 layers of smooth, stratified, non-keratinized squamous epithelium (approximately 50 µm thick) and includes a basement membrane, which consists of the separate layers including lamina lucida and lamina densa referred to in Figure 1.2.

ii. Bowman’s layer: located deep to the basement membrane and consists of a thin layer of Type IV collagen, laminin, heparin sulfate and fibronectin collagen fibrils.

iii. Corneal stroma: 90 % of the corneal thickness, composed of non-cellular and cellular components that maintain the corneal structure.

iv. Descemet’s membrane: thin layer composed of Type IV collagen that serves as a basement membrane for the corneal endothelium.

v. Corneal endothelium: single-cell layer of simple squamous or cuboidal cells that is responsible for regulating transport between the cornea and the aqueous humor.
Figure 1–1 Corneal Anatomy and Histology. (A) Outline of the important anatomical features of the globe with emphasis on the cornea in relation to the other structures. (B) A histological section of the human cornea is shown labeling the five main layers of the cornea (toluidine blue x 25). The histological section has also been edited to demonstrate the corneal nerves (yellow) in the stroma and epithelium. (Modified from Adler’s Physiology of the Eye, Dawson et al. Chapter 4: Cornea and Sclera, page 71 – 130, copyright (2011) with permission from Elsevier: License # 4063201128081)

The corneal epithelium forms the protective outer layer of the cornea and is exposed to the external environment. The epithelial cells are adherent to each other through desmosomes and
tight junctions, which include the proteins ZO-1, JAM-A, occludin, and caludin-1 (Klyce & Crosson 1985; Ban et al. 2003). The superficial surface of the corneal epithelium contains microplicae and microvilli which are covered by a layer of glycocalyx and a 7 μm layer of tear film containing mucinous, aqueous, and lipid layers (Argüeso & Gipson 2001; Dawson et al. 2011).

The glycocalyx serves to hydrate the corneal surface and protect the cornea from infection (Dartt 2011). It is composed of soluble polysaccharides secreted by the lacrimal gland and goblet cells and membrane-spanning polysaccharides, including MUC1, MUC4 and MUC16, secreted by the corneal squamous epithelium (Argüeso & Gipson 2001). The mucous layer of the tear film is secreted by the conjunctival goblet cells and is composed predominantly of MUC5AC (Dartt 2011). Secretion of this mucinous layer occurs in response to noxious stimulation of sensory nerves in the cornea via efferent sympathetic and parasympathetic nerves that innervate the goblet cells (Dartt 2011). The aqueous layer of the tear film is primarily secreted by the lacrimal gland with a smaller contribution from accessory lacrimal glands in the conjunctiva. The lacrimal gland also contains afferent innervation from the trigeminal nerve and efferent innervation from the parasympathetic and sympathetic innervation, which stimulate tear secretion (Dartt 2011). The lacrimal gland fluid is isotonic and contains several proteins including lysozyme, lactoferrin, lacretin, IgA, EGF and surfactant proteins (McKown et al. 2009). The most superficial layer of the tear film, the lipid layer, is secreted by the meibomian glands, which are located at the eyelid margin of the tarsal plate. Together, the layers of the tear film protect the ocular surface from infection and prevent desiccation and friction with closing and opening of the eyelids. Loss of these layers results in dry eye disease which can lead to ocular pain and corneal epithelial ulceration (Perry 2008).

The basement membrane and Bowman’s layer are the interface between the corneal epithelium and the corneal stroma. The basement membrane serves as a scaffold for adherence of the basal corneal epithelium to the underlying Bowman’s Layer and corneal stroma and may also influence migration and differentiation of the corneal epithelium (Torricelli et al. 2013). The basal layer of the corneal epithelium contains hemidesmosomes, which, in combination with anchoring fibrils and anchoring filaments, form the anchoring complex that adheres the corneal epithelium to the underlying basement membrane (Torricelli et al. 2013). The anchoring complex contains three necessary components: an intracellular plaque protein, a transmembrane protein,
and an ECM associated with the basement membrane (Torricelli et al. 2013) (Figure 1-2A). The transmembrane proteins include α6β4 integrin and type XVII collagen, which link the basement membrane with keratin intermediate filaments and laminin (van der Neut et al. 1996; Stepp et al. 1990; Dowling et al. 1996). Collagen VII is the major component of anchoring fibrils, and extend through the underlying stroma to attach to anchoring plaques (Gipson et al. 1987). Expression of proteins associated with the anchoring complex change during wound healing and function as a component of cellular migration during wound healing (Fujikawa et al. 1984; Stepp et al. 1996). Abnormalities of the basement membrane are associated with recurrent corneal erosions (Colville et al. 1997; Resch et al. 2009; Payant et al. 1991) and corneal innervation may be necessary for the expression of proteins necessary to anchor the corneal epithelium to the basement membrane and for migration. Administration of Substance P (SP), which is released from nerve terminals in the corneal epithelium, increases cellular migration in vitro and increases the attachment of corneal epithelial cells to a fibronectin, collagen IV, and laminin matrices (Nishida et al. 1996; Yamada et al. 2005). SP may also improve epithelial cell attachment by increasing E-cadherin expression (Araki-Sasaki et al. 2000) and α5 integrin expression (Chikama et al. 1999), which has been demonstrated in cultured SV-40-transformed human corneal epithelial cells in a dose-dependent manner. While higher mammals, such as humans, have a well-developed Bowman’s layer in comparison to rodents and other species (Hayashi et al. 2002), there are no obvious differences in anchoring fibril structure with or without Bowman’s layer (Gipson et al. 1987).

Transparency of the corneal stroma is maintained by a dense, regular arrangement of collagen fibrils organized into orthogonal lamellae (Hassell & Birk 2010; Jester et al. 1999; Maurice 1957). This structure is maintained by keratocytes, which function as modified fibroblasts and reside in the corneal stroma between the lamellae (Dawson et al. 2011). The cornea contains 13 different types of collagen, with the primary collagen Type I collagen (58 %) (Meek & Fullwood 2001; Komai & Ushiki 1991; Robert et al. 2001). The corneal stroma also contains proteoglycans, which help maintain spatial order of collagen and maintain corneal shape and volume (Dawson et al. 2011).
Figure 1–2 Anchoring Complexes and the Corneal Epithelium. (A) Schematic of the basement membrane is shown, demonstrating the basal cell layer of the corneal epithelium and the hemidesmosome-anchoring filament complex. This is a simplified diagram and does not represent a complete schematic, but shows only selected molecules components. HD, hemidesmosome; BP230,
bullous pemphigoid antigen 230 (Reprinted from Torricelli et al. The corneal epithelial basement membrane: structure, function and disease, page 6393, copyright (2013) with permission from ARVO)

(B) The corneal epithelium is composed of 3 layers, including a single layer of basal epithelium, two to three layers of wing cells, and a single layer of squamous epithelium. The interaction between nerve terminals in the corneal epithelium and the corneal epithelial cells may increase cellular adhesion molecules that improve adhesion of the corneal epithelial cells to each other and the underlying basement membrane (Modified from Adler’s Physiology of the Eye, Dawson et al. Chapter 4: Cornea and Sclera, page 71 – 130, copyright (2011) with permission from Elsevier: License # 4063200740004).

The posterior surface of the cornea is protected by the corneal endothelium and Descemet membrane, which serves as the basement membrane for the corneal endothelium. The corneal endothelium consists of a thin single layer of polygonal cells that play an essential role in regulating stromal dehydration, which is necessary in maintaining corneal clarity (Eghrari et al. 2015). The corneal endothelium and Descemet membrane also function as a leaky barrier, which is essential in maintaining corneal health as most nutrients for the cornea derive from the aqueous humor (Dawson et al. 2011). The endothelium and Descemet membrane are not innervated, as the innervation of the cornea is restricted to the anterior one-third of the corneal stroma and the corneal epithelium.

1.3.3 The Corneal Innervation

The corneal epithelium is the most densely innervated tissue in the body, with a corneal nerve density estimated to be 300 to 600 times more dense than skin (Rozsa & Beuerman 1982; Guthoff et al. 2005). The sensory corneal innervation derives from the ophthalmic branch (V1) of the trigeminal nerve (CN V) which enters the orbit and travels to the cornea by way of the long ciliary nerves (Müller et al. 2003) (Figure 1-3). The results of retrograde nerve tracing studies suggest that, despite the density of corneal innervation, the cornea is supplied by nerves of relatively few trigeminal neurons (50-450) with a single trigeminal neuron supporting hundreds of individual nerve endings (de Castro et al. 1998; De Felipe et al. 1999; LaVail et al. 1993; Marfurt et al. 1989; Ivanusic et al. 2013; Launay et al. 2015). The cornea may also contain sympathetic and parasympathetic fibers that derive from the superior cervical ganglion and the
ciliary ganglion, respectively. Both are believed to be sparse in humans, moderate in rats, and occur in the periphery of the cornea (Marfurt et al. 1993; Toivanen et al. 1987; Marfurt et al. 1998).

Figure 1–3 Corneal Nerve Anatomy. This is a medial view of the orbit demonstrating the path of the corneal innervation. The primary sensory neurons are located in the trigeminal ganglion, and travel to the cornea through the superior orbital fissure via the ophthalmic nerve and long ciliary nerves. (Reprinted from Adler’s Physiology of the Eye, Dawson et al. Chapter 4: Cornea and Sclera, page 71 – 130, copyright (2011) with permission from Elsevier: License # 4063201331037)

The corneal innervation is divided into four levels: i. stromal nerve trunks (SN), ii. sub-epithelial nerve plexus (SEP), iii. sub-basal nerve plexus (SBP), and iv. epithelial nerve terminals (ENT) (Belmonte et al. 2011) (Figure 1-4A). The majority of the corneal innervation via the limbic plexus (LP), enters the anterior corneal stroma radially with the axons shedding their myelin sheaths and perineurium within 1 mm of the limbus (Fig. 1-4B), after which they are covered by only Schwann cell (SC) sheaths (Müller et al. 2003). The SNs branch extensively and
anastomose within the anterior corneal stroma, forming the SEP. Fibers from the SEP penetrate Bowman’s membrane and the basement membrane, dividing into thinner axons that continue as the SBP (Fig. 1-4B). Axons, now devoid of SC coverage, travel parallel to the surface of the cornea creating a characteristic whorl-like pattern of nerve fibers (Belmonte et al. 2011). Single fibers then divide from the sub-basal plexus to travel anteriorly through the corneal epithelium and terminate as free epithelial nerve endings (ENT).

Approximately two-thirds of the sensory fibers innervating the cornea are unmyelinated C fibers that terminate as polymodal nociceptors (Belmonte 1993; Belmonte & Giraldez 1981). Due to the extensive branching of these axons within the cornea, the receptive fields tend to be large with a great deal of overlap (Belmonte et al. 2011). The remaining sensory fibers are cold thermal receptors (10 to 15 %) and mechano-nociceptors (15 to 20 %) which consist of thinly myelinated (Aδ) axons (Belmonte et al. 2011). Intraepithelial nerves terminate as free nerve endings throughout the layers of the corneal epithelium and are most numerous in the wing and basal cell layers (Belmonte et al. 2011). Free nerve endings are associated with invaginations of the epithelial cell membranes, suggesting a possible interaction between the corneal nerves and the corneal epithelium (Müller et al. 2003). The corneal innervation is also a dynamic structure which is known to demonstrate centripetal movement (Patel & McGhee 2008), possibly in conjunction with corneal epithelial migration. Shedding of the corneal epithelial cells may also influence intraepithelial nerve terminal rearrangement (Harris & Purves 1989).
**Figure 1–4 Pattern of the Corneal Innervation.** (A) Schematic of the distribution of the corneal innervation demonstrating the limbal plexus (LP), stromal nerve trunks (SN) that form the subepithelial plexus (SEP), the sub-basal plexus situated beneath the basal epithelium (SBP), which then give rises to intraepithelial nerve terminals (ENT). (B) The cornea contains mostly unmyelinated C-fibers and a small number of myelinated Aδ fibers which lose their myelin sheath shortly after entering the cornea. Subepithelial nerves pierce Bowman’s layer to form the sub-basal nerve plexus (3) that runs parallel to the surface of the cornea and gives off vertical intraepithelial nerve terminals (4), which terminate at various layers of the corneal epithelium. (Modified from Adler’s Physiology of the Eye, Dawson *et al.* Chapter 4: Cornea and Sclera, page 71 – 130, copyright (2011) with permission from Elsevier: License # 4063201331037).
1.3.4 Corneal Epithelial Maintenance and Repair

The entire corneal epithelium is estimated to turn over every 7 to 10 days (Hanna et al. 1961; Cenedella & Fleschner 1990), necessitating a population of epithelial progenitor cells that are capable of proliferation and migration to replace the damaged cells. The predominant theory for corneal epithelial homeostasis is the XYZ hypothesis, proposing that proliferating limbal stem cells (X) are a source of transient amplifying cells (TACs) (Y) which migrate centripetally to continually replenish the corneal epithelial cells that are lost to desquamation (Z) (Thoft & Friend 1983). This hypothesis is based on early studies that observed that centripetal migration of melanin expressing and India Ink labelled cells occurred from the corneal periphery towards the central cornea (Mann 1944; Davanger & Evensen 1971; Pfister & Burstein 1976; Buck 1979; Buck 1985). More recently, these observations have been confirmed using transgenic mice with reporter genes (Collinson 2004; Nagasaki & Zhao 2003) and inducible multicolor genetic tagging (Di Girolamo et al. 2015) to demonstrate the centripetal migration of cells emerging from the basal limbal epithelium. Corneal epithelial cells are thought to migrate at a rate of 120 µm per week under normal circumstances (Buck 1985).

Following corneal epithelial injury or ulceration, wound closure is dependent on the proliferation of limbal stem cells and TACs migrating over the exposed underlying basement membrane and stroma. Epidermal growth factor (EGF) signalling is a major pathway mediating corneal epithelial proliferation and migration (Zieske et al. 2000; Nakamura, Nishida, et al. 1997a; Rush et al. 2014; Winkler et al. 2014; Funari et al. 2013; Peterson et al. 2014) via activation of the phosphatidylinositol-3-kinase (PI3K)-Akt and extracellular regulated kinase (ERK) pathways (Lu et al. 2010; Wang et al. 2012). Corneal innervation may play a role in EGF receptor (EGFR) activation after injury. Rats with type 1 diabetes mellitus, which is known to cause damage to the corneal innervation, show delayed wound healing associated with decreased activation of the EGFR signaling pathway (Xu & Yu 2011) while activation of these pathways with topical substance P (SP), which is released by the corneal nerve terminals, promotes wound healing in rats with type 1 diabetes mellitus (Yang et al. 2014).

Regulation of extracellular matrix proteins, proteinases and other growth factors and cytokines have also been implicated in corneal wound healing (Ljubimov & Saghizadeh 2015), including hepatocyte growth factor (HGF) (Li et al. 1996; Saghizadeh et al. 2011), insulin-like growth
factors (IGFs) (Lee et al. 2006; Trosan et al. 2012), platelet-derived growth factors (PDGFs) (Kamiyama et al. 1998), and nerve growth factor (NGF) (Lambiase et al. 1998; Lambiase et al. 2000; Micera et al. 2006; Blanco-Mezquita et al. 2013). These are reviewed in detail by Muller et al. (2003) and Shaheen et al. (2014).

1.3.5 Characterization of Limbal Stem Cells

Studies suggest that corneal epithelial maintenance is dependent on a distinct population of stem cells located in the basal epithelium at the junction of the cornea and sclera (Schlötzer-Schrehardt & Kruse 2005; Pellegrini et al. 1999), which migrate centripetally to replenish the corneal epithelium (Mann 1944; Davanger & Evensen 1971; Pfister & Burstein 1976; Buck 1979; Buck 1985; Collinson 2004; Nagasaki & Zhao 2003; Di Girolamo et al. 2015). The anatomical location where the cornea meets the sclera is referred to as the limbus. Cells at the limbus are associated with increased frequency of mitoses and demonstrate a higher proliferative potential than cells from the central cornea (Friedenwald & Buschke 1944; Hanna & O’Brien 1961; Ebato et al. 1988; Pellegrini et al. 1997; Pellegrini et al. 1999; Lindberg et al. 1993). Molecules associated with asymmetric cell division have also been exclusively localized to the limbus, including Mushashi-1 (Raji et al. 2007), Notch-1 (Thomas et al. 2007), p75 (Di Girolamo et al. 2008), C/EBPδ (Barbaro et al. 2007), and ΔNp63α (Di Iorio et al. 2005). Cells contained within the limbus reside in a growth-arrested, slow-cycling state (Cotsarelis et al. 1989; Zhao et al. 2009; Pajoohesh-Ganjii et al. 2006; Figueira et al. 2007) and demonstrate cellular morphology characteristic of a stem cell population (Bentley et al. 2007; Chen et al. 2004; Schlötzer-Schrehardt & Kruse 2005; Romano et al. 2003). Limbal cells demonstrate inducible proliferative potential following corneal injury (Cotsarelis et al. 1989; Lehrer et al. 1998). No consensus exists regarding a standard set of cell markers to characterize limbal stem cells, but the markers ABCD2, p63, p75NTR, TrkA and Hes1 are accepted as distinguishing the limbal stem cell population in vivo (Takács et al. 2009; Schlötzer-Schrehardt & Kruse 2005). ABCB5 was also found to characterize limbal stem cells (Frank & Frank 2015; Ksander et al. 2014).

The chemotactic signal for centripetal migration is unknown but may be due to the different composition of the limbal and corneal extracellular matrices, including interleukin-6 (Nishida et
al. 1992), fibronectin (Kimura et al. 2007), and hyaluronan (Nishida et al. 1991; Lu et al. 2001). Corneal denervation has also been shown to reduce corneal limbal stem cell number and diminish function in mice (Ueno et al. 2012), suggesting a role for innervation in inducing limbal cell proliferation or migration. Limbal stem cells express TrkA, and neuromediators, such as NGF, are known to improve expansion of cultured limbal stem cells in vitro (Qi et al. 2007; Qi et al. 2008; Touhami et al. 2002). NGF also improves healing of the corneal epithelium in patients with persistent corneal epithelial defects.
1.4 Neurotrophic Keratopathy

1.4.1 Introduction

This subchapter summarizes the epidemiology and physiology of neurotrophic keratopathy (NK) as well as current trends in the management and how surgical corneal reinnervation addresses some of the limitations of conventional ophthalmologic treatment.

1.4.2 Epidemiology, Diagnosis and Prognosis of Neurotrophic Keratopathy

Corneal sensation is a necessary component of reflexes that protect the cornea from injury and nerve-derived mediators are known to provide trophic support to the corneal epithelium (Ueno et al. 2012; Müller et al. 2003; Dawson et al. 2011). Patients with absent or impaired corneal sensation develop NK, which is a disease characterized by corneal anesthesia or hypoesthesia, occult corneal injury, persistent epithelial ulcerations, stromal scarring and progressive vision loss and blindness. Corneal nerve injury can occur from multiple causes (Table 1.1), including viral infection (herpes simplex and herpes zoster keratoconjunctivitis), chemical burns, intracranial trigeminal nerve injury or corneal surgery. Corneal anesthesia or hypoesthesia can also be congenital (Ramaesh et al. 2007; Mathen et al. 2001) and may be isolated or occur in association with VACTERL (Cruysberg et al. 1998), and Goldehar (Villanueva et al. 2005) or Mobius syndromes (Carta et al. 2011). Systemic diseases that affect the peripheral nervous system, such as diabetes, multiple sclerosis, or leprosy, may also decrease corneal nerve density and sensation resulting in neurotrophic keratopathy (Lockwood et al. 2006).
Table 1–1 Etiology of Neurotrophic Keratopathy

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<td>Goldenhar-Gorlin Syndrome</td>
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<td>Mobius Syndrome</td>
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<td>Familial Corneal Hypoesthesia</td>
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<td>Corneal Dystrophies</td>
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<td>Leprosy</td>
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NK is an orphan disease, affecting fewer than 5 in 10 000 people (Sacchetti & Lambiase 2014). The diagnosis of NK is made clinically and based on: i) clinical findings of corneal hypoesthesia or anesthesia, and ii) history or presence of recurrent or persistent corneal epithelial defects. As patients have absent corneal sensation, corneal injuries typically go unrecognized until scarring of the cornea occurs and causes vision loss, making NK a difficult disease to diagnose early in a primary care setting. Documentation of absent corneal sensation and corneal epithelial
breakdown are necessary to diagnose NK. Corneal sensation can be tested grossly with a cotton swab or more accurately with Cochet-Bonnet esthesiometry (Dawson et al. 2011), which measures sensation by recording the patients response when pressure is applied to the ocular surface using a nylon filament of variable length (0 to 60 mm). More recently, CO₂ gas esthesiometry has also been used to test corneal sensation more accurately (Tesón et al. 2012) and in vivo corneal confocal microscopy (IVCCM) can be used to document the absence of corneal innervation (Villani et al. 2014). The second criterion for the disease, corneal epithelial abnormalities, can be detected with fluorescein dye and slit lamp microscopy. Fluorescein stains the underlying basement membrane and stroma only in the presence of damage to the corneal epithelium.

NK is classified based on the severity of corneal disease into three stages (Mackie 1995):

I. Stage I: characterized by punctate keratopathy, epithelial irregularity and stromal scarring

II. Stage II: defined by persistent corneal epithelial defects with adjacent corneal epithelium that is characterized by loose adherence to the underlying basement membrane.

III. Stage III: corneal epithelial ulcerations that progress to stromal involvement, including possible perforation or stromal melting, which is the progressive dissolving of the stroma.

1.4.3 The Molecular Basis of Neurotrophic Keratopathy

While the corneal nerves are a necessary component of reflexive blinking and tearing, the corneal innervation is also a source of trophic mediators necessary for corneal epithelial maintenance and repair (Rosenberg 1984; Müller et al. 2003). Animal models of NK have demonstrated that the corneal epithelium swells immediately following denervation, with associated loss of characteristic epithelial microvilli and dilation of the intracellular spaces of the basal layers, predisposing the epithelium to damage and accelerated rates of sloughing (Beuerman & Schimmelpfennig 1980; Knyazev et al. 1991). In the absence of corneal innervation, epithelial cells also demonstrate decreased cell vitality and mitosis, resulting in thinning, breakdown and ulceration of the corneal epithelium (Sigelman & Friedenwald 1954; Alper 1975; Cavanagh & Colley 1989; Beuerman & Schimmelpfennig 1980). Corneal
denervation also impairs the ability of the cornea to heal following injury (Beuerman & Schimmelpfennig 1980; Schimmelpfennig & Beuerman 1982; Araki et al. 1994; Gallar et al. 1990; Ferrari et al. 2011), which may in part be due to innervation playing a vital role in limbal stem cell proliferation and migration after corneal epithelial injury (Cavanagh & Colley 1989; Bonini et al. 2003; Sigelman & Friedenwald 1954; Ueno et al. 2012). Co-culture of corneal epithelial cells and dissociated trigeminal neurons have suggested a trophic influence of trigeminal neurons on epithelial differentiation and proliferation (Baker et al. 1993; Chan & Haschke 1982; Chan & Haschke 1981; Kowtharapu et al. 2014; Ko et al. 2013).

Several neuromodulators released from sensory axons innervating the cornea have been proposed as regulators of corneal epithelial maintenance and healing (Shaheen et al. 2014; Müller et al. 2003). A select few factors are discussed here:

**Substance P.** Substance P is present in the mammalian and human cornea (Murphy et al. 2001; Marfurt & Echtenkamp 1995; Elbadri et al. 1991; Tervo & Tervo 1981) and has been shown to decrease in patients with corneal hypoesthesia (Yamada et al. 2000; Stone & Kuwayama 1985). Administration of systemic capsaicin to neonatal rats, which depletes Substance P (SP) from axon terminals expressing TRPV1, a nonselective cation channel expressed on nociceptive neurons, has also been shown to cause corneal changes consistent with NK (Fujita et al. 1984; Shimizu et al. 1987; Donnerer et al. 1996). This suggests that SP is important for the maintenance of the corneal epithelium. SP stimulates corneal epithelial proliferation and may be improve epithelial maintenance and would healing (Reid et al. 1993; Garcia-Hirschfeld et al. 1994) through a cAMP-dependent pathway (Shimizu et al. 1987; Fujita et al. 1984; Cavanagh & Colley 1989; Mikulec & Tanelian 1996; Chikama et al. 1998; Donnerer et al. 1996). *In vitro*, SP enhances EGF stimulation of corneal epithelial cell migration and SP has been shown to increase EGFR activation and to promote corneal epithelial healing in a rat model of diabetic keratopathy (Xu & Yu 2011; Yang et al. 2014; Yin et al. 2011). The SP-receptor, NK-1, is also expressed on the corneal epithelial cells and mediates epithelial responsiveness to SP (Kieselbach et al. 1990; Nakamura, Ofuji, et al. 1997; Mantyh 2002; Yang et al. 2014).

Topical SP has been shown to work synergistically with IGF-1 to heal corneal epithelial ulcerations in a rat model of neurotrophic keratopathy (Nagano et al. 2003) and diabetic keratopathy (Nakamura et al. 2003), as well as *ex vivo* culture of corneal epithelium (Nishida et
al. 1996). SP, in conjunction with insulin-like growth factor–1 (IGF-1), has been shown to increase cell migration (Nishida et al. 1996) and to upregulate the expression of α5 integrins and E-cadherin. The latter is necessary for epithelial adhesion to fibronectin in the extracellular matrix and to maintain the integrity of the corneal epithelium (Nakamura et al. 1998; Chikama et al. 1999; Araki-Sasaki et al. 2000). SP may also play a role in the formation of corneal epithelial tight junctions by increasing the expression of ZO-1 (Ko et al. 2009) in the superficial layer of the corneal epithelium.

SP, SP analogues, and IGF-1 have been used in several case reports to treat persistent epithelial defects (PEDs) in human patients, and have demonstrated improved corneal re-epithelization in most studies (Kingsley & Marfurt 1997; Murphy et al. 2001; Morishige et al. 1999; Chikama et al. 1998; Brown et al. 1997; Nakamura et al. 2003; Yamada et al. 2008). More recently, SP has also been implicated in playing a role in stromal wound healing as well (Sloniecka et al. 2015; Sloniecka et al. 2016).

**Calcitonin-gene related peptide (CGRP).** The cornea is also innervated by nerves containing CGRP (Jones & Marfurt 1991; Marfurt et al. 1998) and a large proportion of corneal nerves contain both SP and CGRP (Marfurt et al. 2001; Beckers et al. 1993). CGRP receptors are also expressed on corneal and limbal epithelial cells (Heino et al. 1995; Tran et al. 2000a) suggesting a role for CGRP in normal corneal epithelial maintenance. CGRP has been shown to increase following corneal wounding (Mertaniemi et al. 1995). However, results following corneal wounding have been mixed with some studies showing enhancement of epithelial cell migration by exogenous CGRP (Mikulec & Tanelian 1996) with no effect found in others (Nishida et al. 1996; Nakamura, Nishida, et al. 1997b). However, in these studies CGRP was not co-administered with other neuromediators such as SP which, when administered together, increased corneal epithelial DNA synthesis (Reid et al. 1993), suggesting that CGRP may only have synergistic effects with other nerve-derived mediators. CGRP may be necessary for differentiation of the corneal epithelial cells (Garcia-Hirschfeld et al. 1994) and both CGRP and SP have been reported to stimulate IL-8 secretion following injury, suggesting an inflammatory role for both substances (Tran et al. 2000a). While CGRP does not appear to influence the corneal epithelium in isolation, it may function to potentiate the effect of other nerve-derived factors, such as SP.
Neurotrophins. Neurotrophins, regulated by cutaneous sensory neurons, are known to be important for maintenance, survival and repair of the skin (Ansel et al. 1996; Burbach et al. 2001). Neurotrophins derived from corneal sensory axons may likewise improve corneal epithelial healing following injury (Kruse & Tseng 1993; Lambiase et al. 1998). NGF, TrkA and p75NTR are known constituents of the normal human and rat cornea (Lambiase et al. 2000; Mearow et al. 1993; You et al. 2001; Di Girolamo et al. 2008). TrkA is expressed throughout the corneal epithelium, including the corneal limbus, while p75NTR expression appears to localize to the limbal basal epithelium (Touhami et al. 2002; Di Girolamo et al. 2008). NGF may regulate the survival and proliferation of the limbal stem cell population which resides within the limbus and p75NTR has been proposed as a marker of limbal stem cells (Touhami et al. 2002). NGF promotes proliferation and differentiation of corneal epithelial cells in vitro (Kruse & Tseng 1993; You et al. 2001) and enhances migration of cells following injury (Murphy et al. 2001).

Several reports have demonstrated that epithelial healing is accelerated following the topical administration of exogenous NGF in animal models and in patients with NK (Lambiase et al. 2000; Kruse & Tseng 1993; Lambiase et al. 1998). While NGF has demonstrated some positive clinical results in treating corneal ulcerations in the anesthetic cornea, it remains ineffective in some patients who continue to progress to vision loss (Lambiase et al. 1998; Lambiase et al. 2000; Tan et al. 2006). Unresponsiveness to NGF in some patients with NK may be due to a complete absence of corneal innervation, while patients that respond well have impaired corneal sensation and hypoesthesia, reflecting a diminished population of cornel nerves. In the presence of diminished but not absent corneal innervation, NGF may enhance axonal regeneration (Donnerer 2003; Donnerer et al. 1996) and the NGF/TrkA interaction may play an important role in guiding axonal growth within the cornea. Transgenic mice overexpressing NGF in the epidermis, demonstrate increased axon density of the epidermis and increased number of sensory neurons in the trigeminal ganglion, demonstrating the NGF plays a role in regulating the level of innervation (Albers et al. 1994). The density of nerve terminals in the cornea is diminished in TrkA (-/-) transgenic mice and there is an absence of characteristic sub-basal nerve fibers (de Castro et al. 1998). While this was attributed to decreased neuronal survival in TrkA (-/-) transgenic mice, this finding was not confirmed by counting the number of surviving neurons in the trigeminal ganglion, and this finding could also be attributed to aberrant axonal growth in TrkA (-/-) transgenic mice.
1.4.4 Management of Neurotrophic Keratopathy

NK is one of the most difficult ocular diseases to treat as it lacks a specific management targeting the underlying pathophysiological cause (reviewed by Sacchetti & Lambiase 2014). Early diagnosis and careful monitoring of patients with NK is necessary to detect and treat corneal ulcerations as, aside from vision loss, patients remain asymptomatic throughout the course of the disease because of absent corneal sensation. Conventional treatment aims to protect the corneal epithelium from injury and to promote healing in order to prevent scarring of the underlying stroma. Initial treatment focuses on preventing desiccation and breakdown of the corneal epithelium with preservative-free artificial tears. Other topical lubricants to provide more sustained protection of the corneal surface include carbomer, methylcellulose or sodium hyaluronate eye drops. In the presence of a corneal epithelial ulceration, non-pharmacologic treatments to protect the corneal surface include a protective corneal or scleral lens (Grey et al. 2012).

In the case of persistent or recurrent corneal ulcerations, surgical options to protect the corneal surface and preserve ocular integrity may be considered. Partial or complete surgical closure of the eyelids (i.e., tarsorrhaphy) protects the cornea from injury, preventing corneal epithelial breakdown from desiccation and desquamation with blinking. Covering the corneal surface with an amniotic membrane transplant is an easy to perform surgical option, which promotes corneal epithelial healing in refractory cases (Khokhar et al. 2005). In the case of corneal stromal injury and possible corneal perforation, pedicled conjunctival flaps can be raised and brought over the corneal surface to cover the corneal ulceration and sutured in place. In the case of smaller corneal perforations (< 3 mm), cyanoacrylate glue can be used to close the defect in conjunction with a surgical procedure to protect the corneal surface (Sacchetti & Lambiase 2014). Larger defects however, may require corneal transplantation to restore the normal corneal architecture, although the success of corneal grafts in this population is poor in the absence of corneal sensation due to poor wound healing and recurrence of epithelial ulcerations resulting in scarring of the corneal graft and vision loss (Jhanji et al. 2011; Lambley et al. 2014). The prosthetic replacement of the ocular surface ecosystem (PROSE) device was developed to protect the native cornea with a prosthetic to maintain corneal shape and clarity. Few patients have had PROSE devices to treat congenital NK. Five-year follow-up data for 121 patients who completed treatment with a PROSE device, documented that 32 patients (26.4 %) stopped wearing the
device, mostly due to discomfort and limited improvement in visual acuity. It remains unclear whether the PROSE device decreases long-term complications from NK (Agranat et al. 2016). This was shown in another study that included patients with NK, in which corneal epithelial defects recurred in all but one patient following cessation of therapy with the PROSE device (Ling et al. 2013).

Current treatments fail to address the underlying cause of NK, i.e., the lack of corneal innervation and corneal nerve mediated trophic support. While surgical treatments are effective at protecting the corneal surface and preserving ocular integrity, they significantly occlude vision resulting in significant patient morbidity. More recent pharmacologic treatments are in development, which focus on restoring the cornea with the trophic mediators normally provided by the corneal innervation. Autologous serum, platelet-rich plasma, and umbilical cord serum have demonstrated complete healing of the corneal epithelium in patients with NK (Soni & Jeng 2016). Specific neuropeptides, such as SP and IGF-1 demonstrated resurfacing in 82% of epithelial corneal ulcerations over four weeks in patients with NK (Yamada et al. 2008). Topical NGF has also demonstrated significant improvement in healing of neurotrophic corneal ulcerations (Lambiase et al. 1998; Ferrari et al. 2014; Bonini et al. 2000), and is currently undergoing Phase II clinical trials (Sacchetti & Lambiase 2014). However, while these treatments may be effective to improve healing of corneal ulcerations once they are identified, patients remain dependent on daily treatment and susceptible to occult injury that may go unrecognized and result in stromal scarring.

Even with optimal ophthalmic management, NK frequently proceeds to corneal scarring and vision loss. Corneal transplantation is unfortunately ineffective in these patients as the graft remains susceptible to injury and scarring in the absence of corneal innervation (Lambley et al. 2014). While significant progress has been made in the treatment of NK, conventional treatment still includes disfiguring surgical procedures to protect the corneal surface and patients remain dependent on the daily use of topical pharmaceuticals. Furthermore, none of these treatments restore sensation to the cornea, making patients dependent on frequent physician follow-up to ensure that the corneal epithelium remains intact and that there is no occult epithelial injury.
1.4.5 Surgical Reinnervation of the Cornea

Surgical repair of the damaged ophthalmic nerve was the first attempt at surgical reinnervation of the neurotrophic cornea (Samii 1981). Through an osteoplastic frontal craniotomy, Samii exposed the anterior cranial fossa and opened the roof of the orbit to expose the damaged ophthalmic nerve (V1) innervating the affected cornea. He then transected the ophthalmic nerve proximally, transferred it outside of the skull and repaired it to an extracranial peripheral nerve (the occipital nerve) via a sural nerve graft. He reported improved vision and ocular surface health after performing the procedure in three patients with NK; however no patient regained normal corneal sensation. An intracranial procedure with associated craniotomy causes significant patient morbidity and as a result this procedure was not adopted by other surgeons.

Neurotization is a procedure in which a healthy donor nerve is transferred to another part of the body to reinnervate the tissue and restore either motor or sensory function. Neurotization is an established surgical treatment for a range of clinical conditions (Narakas & Hentz 1988; Xiao et al. 2014; Kunihiro et al. 1991; Hontanilla et al. 2014; Brunelli 2004; Brunelli & Brunelli 2004). Instead of repairing the injured ophthalmic nerve, which is intracranial and difficult to access, Terzis et al. (Terzis et al. 2009) used two peripheral nerves, the contralateral supratrochlear and supraorbital nerves, to reinnervate (i.e. neurotization of) the cornea by directly transferring them across the face and suturing them adjacent to the cornea of the affected eye (Figure 1-5). Terzis used this technique in six patients with a long history of NK (mean: 7.0 ± 8.56 years), and demonstrated significant improvement in corneal sensation, visual acuity and corneal health in all patients. Terzis’ surgical technique necessitates a bicoronal incision in order to dissect the supratrochlear and supraorbital nerves and provide sufficient length to directly transfer the nerves to the contralateral cornea. While this avoids the intracranial approach to directly repair V1, the surgical dissection is extensive and the bicoronal incision is disfiguring. Very few reports have been published from other centers using her technique to treat NK (Allevi et al. 2014; Jacinto et al. 2016).
Corneal neurotization by Terzis et al. (2009) was performed by dissecting the contralateral supratrochlear nerve and supraorbital nerve through a bicoronal incision. The two nerves were then transferred to the contralateral face, where they were tunneled into the corneal conjunctiva and (B) sutured adjacent to the corneal limbus (black arrows) to reinnervate the eye. The expectation is that the donor nerves then grow into the cornea to reinnervate the tissue. (Modified from Terzis et al Corneal neurotization: a novel solution to neurotrophic keratopathy, page 112 – 120, copyright (2009) with permission from Wolters Kluwer Health, Inc: License # 4063850714446).
More recently, Borschel and Ali at the Hospital for Sick Children modified Terzis’ technique by using a cross-face nerve graft (CFNG) to obviate the need for a bicoronal incision (Bains et al. 2015; Elbaz et al. 2014). They used two small supraorbital incisions to access the proximal portion of the contralateral supratrochlear nerve, which they then transected proximally and coapted it to a sural nerve graft harvested from the lower leg. The free end of the sural nerve graft was then tunneled subcutaneously and into the contralateral eye (Figure 1-6). Similar to Terzis’ technique, they then dissected the nerve graft into its separate fascicles and transplanted these directly into the corneal stroma. This technique eliminated the need for a bicoronal incision and extensive dissection of the supratrochlear and supraorbital nerves distally, reducing the associated morbidity with the procedure. While this requires an additional sural nerve graft harvested from the lower leg, sural nerve graft harvest is associated with minimal donor site morbidity and is well tolerated in adults (Hallgren et al. 2013; Miloro & Stoner 2005) and pediatric patients (Lapid et al. 2007).

Terzis commented that the time required to re-establish objective corneal sensation in her patient population was 2.80 ± 2.17 years (mean ± SD), which contrasts significantly with Borschel and Ali who demonstrated improved sensation in some patients at three months, and two patients demonstrating corneal sensation indistinguishable from normal (60 mm) within 8 months of corneal neurotization. After 16 ± 2.4 years (mean ± SD) of follow-up, Terzis demonstrated sensation of ≥ 50 mm in only one patient, which again contrasts with Borschel and Ali who demonstrated corneal sensation of 50 mm or greater in three of four treated eyes. Possible explanations for the quicker corneal reinnervation by Borschel and Ali may be explained by the younger patient age, surgical technique and the number of axons present in the donor nerve at the point of transection.

Terzis transplanted the distal nerve into the conjunctiva several millimeters from the corneal limbus, while Borschel and Ali directly transplanted the nerve grafts into the corneal stroma through a stromal pocket. Transplantation of regenerating axons directly into the corneal stroma may maximize the number of donor axons directed towards the cornea and overcome the corneal limbus as a potential barrier to axon regeneration into the cornea (Ahmad et al. 2010; Hertsenberg & Funderburgh 2015; Ferrari et al. 2013). Furthermore, Terzis transected the supratrochlear nerve and supraorbital nerve distally in order to have sufficient length to transfer them to the contralateral side. Nerve caliber decreases distally, reflecting a decreasing number of
donor axons. While the supraorbital nerve contains 6017 ± 4195 axons (mean ± SD) at the orbital rim, fewer than 1620 ± 1045 (mean ± SD) axons are found 6 cm distal to the rim, reflecting a sharp decline in the number of donor axons available distally (Snyder-Warwick et al. 2016). Direct transfer of the supraorbital and supratrochlear nerves requires approximately 10 cm of length. Using a sural nerve graft also has the other advantage of increasing the number of potential donor sources of innervation. Nerve grafts permit the use of nerves that are located too far from the orbit for direct transfer to the corneal stroma.

**Figure 1–6 Corneal Reinnervation Using Nerve Grafts** (A) Corneal neurotization by Borschel and Ali is performed by dissecting the contralateral supratrochlear nerve through a supraorbital incision. The donor supratrochlear nerve is then coapted to the sural nerve graft, which is tunneled into the contralateral conjunctiva. The sural nerve graft provides a pathway for regenerating axons to reinnervate the cornea. (Modified from Bains et al Corneal neurotization from the supratrochlear nerve with sural
1.5 Peripheral Repair and Regeneration

1.5.1 Introduction

Aspects of peripheral nerve anatomy and the physiology of peripheral nerve injury and axon regeneration are summarized, including challenges in optimizing recovery after peripheral nerve repair. These chapters are pertinent to the understanding of axon regeneration into the cornea and they provide the rationale for the studies that are described in Chapter 5.

1.5.2 Peripheral Nerve Anatomy and Physiology

The functional unit of the peripheral nerve is the neuron, which is responsible for communication between the central nervous system and peripheral end-organs. Input from the somatosensory receptors in the periphery mediate interaction with the environment and provide protection from noxious stimuli by relaying information via the conduction of action potentials along the axon. The cell bodies of first-order neurons are located in the dorsal root ganglion or somatic afferent ganglion in the case of cranial nerves, such as the trigeminal ganglion (or Gasserian ganglion) for CN V (Waxman 2011). These then communicate with second order neurons, located in the spinal cord or brainstem, and then finally with the third order neurons which lie in the thalamus and communicate with the sensory cortex (Waxman 2011). Different types of sensory modalities are mediated by a single type of sensory receptor (Table 1-2) (modified from Lundborg 2004; Martin 2012).

Most axons are unmyelinated C-fibers, accounting for approximately 75 % of the total axon number in a cutaneous nerve and 50 % of axons in a motor nerve (Lundborg 2004). The majority of C-fibers terminate as polymodal nociceptors that respond to a variety of mechanical stimuli and temperature. Unmyelinated C-fibers contribute to protective sensibility, producing a slow, burning sensation of pain (Bessou & Perl 1969). Larger diameter fibers are myelinated, increasing the conduction speed of action potentials by decreasing capacitance and restricting action potentials to short unmyelinated segments (nodes of Ranvier), permitting salutatory conduction from node to node (Bean 2007; Rushton 1951). In the peripheral nervous system,
myelin is a lipid rich elongated, flattened extension of the SC membrane which ensheaths the peripheral axons in concentric layers (Nave & Werner 2014). SCs myelinate axonal internodes in a 1:1 ratio, however multiple unmyelinated axons can be ensheathed by a single SC, with the entire assembly referred to as a Remak bundle (Brushart 2011).

Table 1–2 Sensory Modality and Receptor Type.

<table>
<thead>
<tr>
<th>Sensory Modality</th>
<th>Receptor Type</th>
<th>Axon Diameter (μm)</th>
<th>Group</th>
<th>Myelination</th>
<th>Conduction (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Touch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Texture/Superficial</td>
<td>Meissner's and Merkel's</td>
<td>6 - 12</td>
<td>Aβ</td>
<td>Myelinated</td>
<td>12 - 30</td>
</tr>
<tr>
<td>Deep Pressure</td>
<td>Mechanoreceptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibration</td>
<td>Pacinian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensual Touch</td>
<td>Mechanoreceptors</td>
<td>0.2 - 1.5</td>
<td>C</td>
<td>Unmyelinated</td>
<td>0.5 - 2</td>
</tr>
<tr>
<td>Limb Proprioception</td>
<td>Static/Dynamic</td>
<td>13 - 20 ; 6 - 12</td>
<td>Aγ,Aβ</td>
<td>Myelinated</td>
<td>70 - 120 ;15 - 30</td>
</tr>
<tr>
<td>Thermal Sense</td>
<td>Cold receptors</td>
<td>1 - 5</td>
<td>Aδ</td>
<td>Myelinated</td>
<td>12 - 30</td>
</tr>
<tr>
<td></td>
<td>Warmth receptors</td>
<td>0.2 - 1.5</td>
<td>C</td>
<td>Unmyelinated</td>
<td>0.5 - 2</td>
</tr>
<tr>
<td>Pain</td>
<td>Nociceptors</td>
<td>1 - 5</td>
<td>Aδ</td>
<td>Myelinated</td>
<td>12 - 30</td>
</tr>
<tr>
<td>Sharp (fast)</td>
<td></td>
<td>0.2 - 1.5</td>
<td>C</td>
<td>Unmyelinated</td>
<td>0.5 - 2</td>
</tr>
<tr>
<td>Dull (slow)</td>
<td>Pruritic receptor</td>
<td>0.2 - 1.5</td>
<td>C</td>
<td>Unmyelinated</td>
<td>0.5 - 2</td>
</tr>
</tbody>
</table>

SCs and axons express various receptors and factors that mediate their interaction, including myelination (Lopez-Leal & Court 2016; Fu & Gordon 1997). The abaxonal membrane, which is the portion of the SC membrane interacting with the endoneurial tubules and the basal lamina of the ECM, expresses several receptors that interact the basal lamina, including α6β4 integrin, dystroglycan, and heparin sulfate proteoglycans (HSPGs), such as perlecan, N-syndecan and glypican (Brushart 2011; Chernousov et al. 2008). Axon contact with SCs influences receptor expression, including the upregulation of the α6β4 integrin that facilitates SC adhesion to laminin (Einheber et al. 1993; Salzer 2003). The abaxonal membrane interacts directly with the axonal membrane, influencing myelination and the expression of myelin-associated glycoprotein (MAG), and several other receptors, including NECL-1, NECL-2, Nogo-66 and p75 which mediate SC-axon interactions (Quarles 2002; Spiegel et al. 2007; Birchmeier & Nave 2008; Maurel et al. 2007).

Nerve structure and axon organization are maintained and protected by several layers of connective tissue that are divided into three distinct layers: the epineurium, perineurium and
endoneurium. The epineurium is the connective tissue layer that surrounds the entire outer layer of the nerve, as well as the individual fascicles, while the perineurium contains the individual fascicles. The endoneurium is the ECM that surrounds individual nerve fibers and interacts directly with SCs. Within the connective tissue layers is a robust intraneural vascular system, with interconnections between the epineural, perineurial and endoneurial microvascular systems. The nerve is supplied segmentally by regional vessels, which then supply the epineurial, perineurial and endoneurial plexi. This system is capable of maintaining intraneural circulation despite transection or mobilization during repair for a distance of up to 6 to 8 cm in the absence of tension (Lundborg 2004).

1.5.3 Pathophysiology of Peripheral Nerve Injury

1.5.3.1 Classification of Nerve Injuries

Connective tissue of the peripheral nerves and the undulating course of axons provides some resistance to compressive and elastic forces on the nerve (Sunderland 1990). Despite this, peripheral nerves remain susceptible to injury due to acute or chronic compression and stretch or direct trauma. Peripheral nerve injuries are classified by damage to the major components of the peripheral nerve (Sunderland 1990), with increasing injury severity resulting in poorer axon regeneration:

i. Class I: Transient conduction block typically secondary to myelin disruption from compression or ischemia. Recovery is spontaneous and complete.

ii. Class II: Disruption of axonal continuity resulting in distal Wallerian degeneration.

iii. Class III: Disruption of both the axon and endoneurial tubule, resulting in disordered axonal regeneration through intrafascicular scar tissue.

iv. Class IV: Disruption of the axon, endoneurium and perineurium with only the epineurium remaining intact. Often requires resection of intraneural scar formation and approximation of the nerve ends with surgical repair.
v. Class V: Complete anatomic dissociation of the nerve with dissociation of the nerve segments proximal and distal to the nerve injury.


1.5.3.2 Neuronal Changes after Nerve Injury and Wallerian Degeneration

Following peripheral nerve injury, the neuronal cell bodies undergo morphologic and molecular changes that promote axon regeneration and switch the neuron from a “transmitting” to “growing” state (Fu & Gordon 1997b). This phenotypic change is necessary for distal axon regeneration, the importance of which was first recognized when a conditioning lesion was shown to accelerate axon regrowth after a more proximal injury (McQuarrie & Grafstein 1973). These changes include dissolution of the Nissl bodies, nuclear eccentricity, and nucleolar enlargement (Fu & Gordon 1997b), which correspond to an increase in protein and mRNA synthesis (Cragg 1970; Watson 1974), including an upregulation of growth-associated protein (GAP-43) and cytoskeletal proteins, such as actin and tubulin (Tetzlaff et al. 1988; Tetzlaff et al. 1991; Haas et al. 1990; Haas et al. 1993; Friedman et al. 1995). The injured neurons also release trophic cytokines for SCs and modulate the synthesis of neurotrophins and ECM, contributing to axon regeneration (Boyd & Gordon 2003; Fu & Gordon 1997b). These changes are reviewed in detail by Fu and Gordon (1997).

Degradation of the axon distal to the site of injury occurs as an active process known as Wallerian degeneration. Wallerian degeneration is an essential component of axon regeneration following injury. SCs in the distal nerve stump contribute to phagocytosis of axon and myelin debris, and secrete chemotactic factors, such as IL-1 and MCP-1, that recruit macrophages that contribute to Wallerian degeneration (Chen et al. 2007; Fu & Gordon 1997). Recruited macrophages then, in turn, secrete cytokines that promote phenotypic changes in SCs to a non-myelinating, growth supportive state (DeFrancesco-Lisowitz et al. 2015).
1.5.3.3 The Regenerative Role of Schwann Cells

SCs adopt one of two phenotypes in mature nerves: either a non-myelinating phenotype, where the SCs ensheath multiple small-diameter unmyelinated axons (forming a Remak bundle), or a myelinating phenotype in which they express the promyelinating signal molecule neuregulin (Nave & Werner 2014). After injury, SCs play a pivotal role in axon regeneration with axon regeneration failing to occur in the absence of SC proliferation (Hall 1986). Within hours of axonal injury, SCs distal to the injury site de-differentiate and down regulate genes supporting myelination (reviewed by Stoll and Muller, 1999), such as Krox-20, and express c-Jun (Salzer 2008) and secrete proinflammatory cytokines, including monocyte chemoattractant protein-1α (MCP-1α), and later IL-1β which attracts circulating macrophages (Taskinen & Roytta 2000; Tofaris et al. 2002; Shamash et al. 2002; Martini et al. 2008). These cytokines stimulate the release of phospholipase A$_2$ (PLA$_2$) (López-Vales et al. 2008) and matrix metalloproteinase-9 (MMP-9) (Chattopadhyay et al. 2007) that contribute to myelin breakdown. As SCs lose contact with degenerating axons, they proliferate and dedifferentiate (Jessen & Mirsky 2016).

Proliferating SCs in the distal stump form the bands of Bungner that in conjunction with the basal lamina provide a scaffold for regenerating axons to attach and extend (Ide 1996; Fu & Gordon 1997b; Navarro et al. 2007). Dedifferentiated SCs express glial fibrillary acidic protein (GFAP), neuregulin, Erb2/3, integrins, and neurotrophic factors, including NGF, glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF) and others, which create a growth supportive environment within the endoneurial tubules (Boyd & Gordon 2003b). Differences in growth factor expression by SCs in motor and sensory tracks are important for selective reinnervation of motor and sensory nerve fibers in addition to their support of axon regeneration (Höke et al. 2006; Brushart 1988; Brushart 1993).

The leading edge of axon regeneration, the growth cone, also modulates SC phenotype via neuregulin, which is expressed in axonal growth cones (Carroll et al. 1997; Vartanian et al. 1997; Buonanno & Fischbach 2001). Neuregulin stimulates upregulation of NGF, BDNF, neurotrophin-4/5, and glia-derived neurotrophic factor (GDNF) by SCs which are important for neuronal survival and axon growth (Chen et al. 2007; Fu & Gordon 1997). Upregulation of actin, tubulin and neurofilament are also central to the process of elongation (Tetzlaff et al. 1988). During axon growth, the axon develops anchoring connections with the SCs via adhesion
molecules including L1, neural cell adhesion molecule (NCAM) and tenascin (Martini et al., 2008) that are necessary for axons to elongate through the endoneurial tubule towards denervated end-organs.

### 1.5.4 Principles of Surgical Management after Nerve Injury

Peripheral nerve injuries are managed by surgically reestablishing continuity of the nerve with the aim of maximizing the number of axons that successfully sprout across the repair site. With that in mind, surgical intervention is reserved for patients where nerve continuity has been disrupted (i.e. Sunderland Class V and VI injuries). Microsurgical end-to-end nerve repair is the gold-standard in managing nerve injuries (Diao & Vannuyen 2000; Sanapanich et al. 2002). Tension free-repair improves axon regeneration after surgical repair, and if this is not possible with primary repair then an interpositional nerve graft can be used (Diao & Vannuyen 2000). Several options for nerve grafting exist, including autologous nerve graft, allograft, acellular nerve graft, or other conduits including biologic or synthetic materials (Safa & Buncke 2016). Autologous nerve grafts are preferred for nerve reconstruction because in addition to providing a scaffold with appropriately sized endoneurial channels, they include SCs which promote axon regeneration distally (Farber et al. 2016). Acellular nerve grafts (ANAs) are derived from cadaveric nerve tissue that has been processed to remove all cellular debris, decreasing the immunogenicity of the graft permitting use without the need of immunosuppression (Isaacs & Browne 2014). While ANAs save the patient from a second site of morbidity, research suggests there is poor axon regeneration through ANAs over 3 cm in length (Saheb-Al-Zamani et al. 2013).

### 1.5.5 Factors Limiting Recovery After Repair

Peripheral nerve injuries are devastating for patients, resulting in permanent numbness, paralysis and pain (Missios et al. 2014; Bekelis et al. 2014). While axons in the peripheral nervous system have the ability to regenerate after injury, recovery is incomplete even with optimal surgical management (Kim, Cho, et al. 2003; Kim, Han, et al. 2003; Fu & Gordon 1997b). The window of opportunity for nerve regeneration is short, as the capacity for regeneration declines with time.
and distance, accounting for the particularly poor recovery when nerve injuries occur far from the site of target reinnervation (Fu & Gordon 1995; Gordon et al. 2011; Sulaiman et al. 2002; Gordon 2015). Overcoming these challenges is essential to maximize recovery after peripheral nerve injury and repair, including axon regeneration through nerve grafts when used for sensory and motor reconstruction.

The slow pace of axon regrowth (~ 1 mm/day in humans) and the delayed growth of axons across the surgical coaptation site contribute to prolonged denervation of nerve distal to the site of injury (Fu & Gordon 1995). While the distal stump is transiently supportive of regeneration, expression of regeneration associated genes decreases as the stump becomes chronically denervated (Al-Majed et al. 2000; Chen et al. 2005; Deshpande et al. 2006; Höke et al. 2006; You et al. 1997). Axons forced to regenerate over long distances do so poorly, with few axons successfully traversing long distance to reinnervate targets (Fu & Gordon 1995; Vuorinen et al. 1995; Siironen et al. 1995). Progressive muscle atrophy and degeneration of the neuromuscular junction with time further limits muscle recovery (Anzil & Wernig 1989; Fu & Gordon 1995). Several studies have documented that earlier nerve repair significantly improves recovery (Sunderland 1990; Mackinnon 1989; Millesi 1990; Fu & Gordon 1997b). Despite these challenges, aside from improved microsurgical techniques and early nerve repair, there have been no surgical or pharmacologic treatments clinically widely adopted to accelerate axon regeneration and improve recovery after nerve injury.

1.5.5.1 Retrograde Neuronal Death

An overlooked component of disability limiting recovery may be the retrograde death of motor and sensory neurons following injury (Lowrie & Vrbová 1992; Oppenheim 1991). Loss of functioning neuronal populations precludes axonal regeneration and improving neuronal survival may increase the regenerative capacity of the peripheral nervous system by rescuing neurons necessary for axonal regeneration (Oppenheim 1996; Lowrie & Vrbová 1992). Neuronal pruning occurs as a component of the developing nervous system, as neurons that do not achieve functional connections with end-organs are removed from the system (Oppenheim 1991). Following peripheral nerve injury, motoneuron death has been shown to occur in up to 80% of the neurons supplying a given injured nerve (Ekström et al. 1998; Ma et al. 2001; Schmalbruch
1984; Burls et al. 1991; Rossiter et al. 1996), with the greatest neuronal loss occurring when injuries are proximal (Hu & McLachlan 2003; Ma et al. 2001; Terenghi 1999), occur at a young age (Burls et al. 1991; Koliatsos & Price 1994), and increase in severity (Fu & Gordon 1997b; Ygge 1989; Snider et al. 1992). Neurons appear to be most susceptible to death during the neonatal period and retrograde neuronal death has been demonstrated in several animal models including cats (Risling, Aldskogius & Hildebrand 1983; Carlson et al. 1979), rodents (Schmalbruch 1987a; Schmalbruch 1984; Lavelle & Lavelle 1958; Burls et al. 1991; Lowrie et al. 1987), and monkeys (Liss et al. 1996; Liss & Wiberg 1997). More recently, sensory neuron loss has been implicated as a component of disability following adult human peripheral nerve injury (West et al. 2013; Suzuki et al. 1993). Furthermore, animal studies have demonstrated that neuronal loss is greatest when immediate reinnervation is prevented for a long period of time (Crews & Wigston 1990; Kashihara et al. 1987; Ma et al. 2003).

Neuroprotective treatments directed at preventing retrograde neuronal death may increase the regenerative capacity of the nervous system. Significant neuronal death has long been known to occur in animal models following neonatal nerve injury and recently sensory neuron death following peripheral nerve injuries has been demonstrated in adult patients (Lowrie & Vrbová 1992; Bahadori et al. 2001; Terenghi et al. 2011a). Several agents have been shown to increase motoneuron survival after injury in animal models, including NMDA antagonists (Dick et al. 1995; Cabaj & Slawinska 2012; Nógrádi et al. 2007; Petsanis et al. 2012), magnesium (Gougoulias et al. 2004; Kapoukranidou et al. 2005), ciliary neurotrophic factor (CTNF) (Yuan et al. 2000; Tan et al. 1996), GDNF (Oppenheim et al. 1995; Morcuende et al. 2013; Baumgartner & Shine 1998; Aszmann et al. 2004; Aszmann et al. 2002), BDNF (Sagot et al. 1998; Eriksson et al. 1994) and caspase inhibitors (Chan et al. 2003).

1.5.5.1.1 N-acetyl cysteine and acetyl-L-carnitine

Sensory neurons in the dorsal root ganglia (DRG) are more susceptible to retrograde neuronal death than spinal motor neurons (Hu & McLachlan 2003; Ma et al. 2001; Wiberg et al. 1999). In adult animals, significant sensory neuron loss occurs within one week of nerve injury (Groves et al. 1997; McKay Hart et al. 2002) and increases reaching 35 – 40% of sensory neurons two months after injury (Hart et al. 2002). Neuronal death is mostly apoptotic in nature and regulated
at the mitochondrial level by pro-survival Bcl-2 and pro-apoptotic Bax (Gillardon et al. 1996; Adams & Cory 2007; Hockenbery et al. 1993). In a rat model of peripheral nerve injury, survival of muscle afferent neurons was associated with downregulation of Bax and caspase-3 mRNA (Reid et al. 2009). Cutaneous sensory neurons were significantly more susceptible to retrograde neuronal death and upregulated caspase-3 and Bax relative to Bcl-2 after peripheral nerve injury (Reid et al. 2009).

The antioxidants acetyl-L-carnitine (ALC) and N-acetyl cysteine (NAC) have both been investigated as neuroprotective agents to reduce retrograde sensory and motor neuron death after peripheral nerve injury. NAC has demonstrated a neuroprotective effect on sensory neurons after adult sciatic nerve transection injury (Hart et al. 2004) and on motor neurons following root avulsion and ventral rhizotomy (Zhang et al. 2005). Treatment with 150 mg/kg/day of NAC reduced sensory neuron loss to 5 % after injury (West et al. 2007). Higher doses of NAC, 750 mg/kg/day, were found to be most effective for motor neuron survival when the mechanism of injury was most severe and resulted in greater retrograde neuronal death (Zhang et al. 2005). ALC administration after sciatic nerve injury in adult rats also decreased retrograde neuronal death of sensory neurons after injury and was found to be effective at doses of 50 mg/kg/day, while lower doses rescued fewer neurons (Hart et al. 2002; Wilson et al. 2003). ALC was found to also rescue sensory neurons when administration was delayed for 24 hours after injury (Wilson et al. 2007). ALC has not demonstrated any rescue effect on motor neurons.

Recently, another small neuroprotective compound, P7C3, demonstrated increased motor and sensory neuron survival in a neonatal model of sciatic nerve crush injury in our lab (Kemp et al. 2015). P7C3 is unapproved for clinical use, as are many of the other compounds investigated to protect motor and sensory neurons from retrograde neuronal death. While NAC and ALC have demonstrated motor and sensory neuron survival in adult models of peripheral nerve injury, it is unknown whether they also protect neurons after neonatal injury, as sensory and motor neurons are more susceptible to retrograde neuronal death after neonatal nerve injury.
1.6 Animal Models of Neurotrophic Keratopathy and Corneal Reinnervation

1.6.1 Introduction

Animal models of corneal nerve injury and regeneration are summarized; including the limitations of each model. These chapters are pertinent to the further investigation of corneal neurotization in an animal model of NK (Chapters 3 and 4).

1.6.2 Animal Models of Neurotrophic Keratopathy

The corneal innervation derives from the ophthalmic branch of the trigeminal nerve (CN V). The ophthalmic nerve enters the orbit through the superior orbit fissure, and gives off the long ciliary nerves, which pierce the posterior part of the sclera and travel anteriorly to innervate the cornea (Figure 1-3). The intracranial course of the corneal innervation makes it difficult to access under direct visualization in order to denervate the cornea as an experimental model of neurotrophic keratopathy.

Several models of NK have been developed in different species by injuring the corneal innervation to produce complete corneal denervation. Zander and Weddell (1951) investigated the reaction of corneal nerve fibers to injury using several techniques, including corneal autografting, transection of the ciliary nerves, extirpation of the trigeminal ganglion, as well as transection of the superior cervical sympathetic ganglion in rabbits and macaque monkeys. They concluded that the entire corneal innervation derived from the trigeminal ganglion with no contribution from the infra-orbital nerve or superior cervical sympathetic ganglion. Ciliary nerve transection was found to completely denervate the cornea; however, the induction of corneal changes consistent with NK were so profound that animals had to be sacrificed three days afterwards. Following extirpation of the trigeminal ganglion, the cornea was protected with a permanent tarsorrhaphy and animals were observed for three weeks. The surgical procedure to visualize the trigeminal ganglion required removal of a portion of the temporal lobe. Only half the rabbits survived, however those that did demonstrated complete corneal denervation with a
small number of fibers regenerating into the cornea after three weeks. Corneal reinnervation was suspected to derive from the sympathetic innervation from the conjunctival vasculature. An intracranial approach was also attempted in rhesus monkeys with similar results (Alper 1975).

Intracranial transection of the trigeminal ganglion through either an intradural or extradural approach proved ineffective in rats resulting in complete mortality 24 hours after the procedure due to intracranial bleeding from vessels in close proximity to the trigeminal ganglion (Sigelman & Friedenwald 1954). This subsequently led to the development of a transpalatal approach to damage the ophthalmic nerve (V1) in rats using diathermy cauterization through an intraoral approach through the roof of the mouth. The optimal location of needle penetration was 32 to 48 mm posterior to the demarcation of the hard and soft palate, 32 mm lateral to midline, and 4 mm deep. After some practice, they reported the success of corneal denervation, as assessed by blink reflex, was approximately 60%. Rat corneas were assessed three days after coagulation of V1, and no long-term studies were done to assess corneal reinnervation. A similar technique has been reported in rats (Keen et al. 1982) and rabbits (Schimmelpfennig & Beuerman 1982) with improved success using transpalatal coagulation of the medial trigeminal ganglion.

Treatment of neonatal animals with systemic or subcutaneous capsaicin has also been shown to impair corneal sensitivity and produce a syndrome similar to neurotrophic keratopathy, including the development of ulcerative lesions, neovascularization, and corneal opacification (Keen et al. 1982; Fujita et al. 1984; Ogilvy et al. 1991). Capsaicin stimulates nociceptive nerve endings through intracellular binding to the transient receptor potential vanilloid type 1 (TRPV1), expressed on A-delta and C sensory fibers that terminate as unmyelinated nerve endings within the corneal epithelium (Zander & Weddell 1951; Tervo & Tervo 1981; Rozsa & Beuerman 1982). TRPV1 is a non-selective cation channel, with activation leading to the influx of sodium and calcium resulting in cellular depolarization (Rosenbaum & Simon 2007). Capsaicin-induced stimulation of TRPV1 in neonatal rats induces retrograde neuronal death of sensory neurons in the trigeminal ganglia (Hiura et al. 2002). Topical application of capsaicin has demonstrated long-lasting desensitization of all corneal C-polymodal nociceptors and some of the A-delta nociceptors (Gallar et al. 1990). However, capsaicin seems to have no effect on nonspecific acetylcholinesterase (NsAchE) positive nerves in the cornea, which are believed to have a functional property other than chemical nociception (Hiura & Nakagawa 2004; Nakagawa et al. 2009). Keen et al. (1982) demonstrated reduced corneal sensation in only 65% of rats after the
Application of topical capsaicin while all rats maintained a corneal reflex, suggesting persistent corneal innervation. In contrast, all rats that had been sensorily denervated by transection of the ophthalmic nerve demonstrated no corneal reflex. In a separate study, topical (3.3 mM) and retrobulbar (33 mM) capsaicin in conjunction, but not in isolation, delayed corneal healing (Gallar et al. 1990). However, no investigations were performed to investigate corneal nerve density afterwards to confirm that the cornea had been completely denervated.

Following neonatal capsaicin administration, retrograde labeling of neurons innervating the cornea has demonstrated a decline, however rats have demonstrated a higher nerve fiber density in comparison to control rats that were treated with saline, suggesting compensatory sprouting of remaining neural fibers in response to partial denervation by capsaicin (Ogilvy et al. 1991; Marfurt et al. 1993; Hiura & Nakagawa 2004; Hiura & Nakagawa 2005). While capsaicin is a minimally invasive means of corneal denervation that avoids ocular damage, the treated rats appeared to retain some degree of sensation and the sprouting of the remaining axons, limiting the utilization of nerve density as an outcome measure following surgical reinnervation of the cornea.

Tervo et al. (1979) was the first to describe a transcranial stereotactic approach to the ophthalmic nerve in adult Sprague-Dawley rats through a burr hole 9 mm anterior to the interauricular line, 2 mm lateral to midline and lowered a coagulation needle to a point between 0.5 and 1.0 mm from the skull base. Ophthalmic nerve ablation was observed to effectively ablate the cornea up to 8 days afterwards, in contrast to ciliary and superior cervical ganglioectomy, which again demonstrated failure to decrease corneal innervation density (Tervo et al. 1979). Stereotactic ablation of V1 has also been successfully performed using radiofrequency by others using coordinates calculated from bregma, which is the intersection of the coronal and sagittal sutures. Radiofrequency and controlled regulation of the tip temperature on the ablative needle, 60° for 105 seconds, has been used to successfully coagulate the ophthalmic nerve (Nagano et al. 2003). Stereotactic surgery has also been described using a transpalatal ventral approach to the ophthalmic nerve (Wong et al. 2004). Wong et al. also utilized temperature controlled radiofrequency of 80° for 4 minutes. In both studies, corneal denervation was documented as an absent blink reflex with confirmed damage to V1. However, neither study actually investigated the presence of corneal nerves after stereotactic surgery.
Transcranial stereotactic ablation of V1 has also been described in mice using electrocautery to coagulate V1 as opposed to temperature-controlled radiofrequency (Ferrari et al. 2011). A conductive, insulated unimodal electrode was lowered through the brain towards V1 after a small burr hole was made at three separate locations. At each location a 2-mA current was passed for 15 seconds. Successful ablation was confirmed as an absent blink reflex. Mice demonstrated rapid onset of the clinical signs of NK, with 90 % of mice demonstrating effective corneal denervation without other neurologic complications, demonstrating a high degree of success with intracranial electrocautery. While the study documented corneal denervation after injury, the purpose of the article was to investigate decreased epithelial proliferation and cellular apoptosis after corneal denervation, and therefore there was no quantification of corneal nerve density before and after V1 ablation.

While stereotactic surgery has been shown to be effective for corneal denervation, and has the advantage of leaving the corneal surface untouched, these techniques are blind, preventing visual confirmation of V1 injury, and require specialized equipment and exposure of the intracranial cavity, which may increase animal mortality. For these reasons, animal models of NK have been described using lateral conjuctival incisions to transect the long ciliary nerves (Yamaguchi, Turhan, Deshea L. Harris, et al. 2013). Transection of the ciliary nerves resulted in near complete corneal denervation at two weeks in 95.8 % of treated rats. However, corneal reinnervation past two weeks was not examined. In personal communication with the authors they explained that nerve regeneration into the cornea occurred at time points longer than two weeks.

Trephination of the cornea has also been performed to radially transect all nerves innervating the central cornea in a rabbit model (Chan-Ling et al. 1987). However this technique results in only partial denervation of the cornea and permits spontaneous regeneration of the corneal nerves, which remain intact in the peripheral cornea, precluding it as a suitable animal model to study corneal reinnervation from an alternative source.
1.6.3 Animal Models of Corneal Reinnervation and Neurotization

While a technique to reinnervate the cornea was first reported by Samii in 1981, due to the invasive nature of the procedure, corneal reinnervation in patients with NK was not really considered a possibility until Terzis’ published the results of her technique in 2009. The second publication investigating corneal reinnervation did not occur until Borschel and Ali’s modification of the technique was published 5 years later in 2014. As surgical reinnervation of the cornea has only recently been adopted as a treatment clinically, there exist no laboratory work investigating how corneal reinnervation from a foreign donor nerve influences the corneal epithelium.

Presently, animal models of corneal reinnervation are used to exclusively investigate regeneration of the native corneal innervation using animal models in which the corneal nerves are damaged within the cornea but there remains some intact native innervation. These models are used to investigate corneal nerve regeneration after damage to the corneal innervation from infectious and metabolic diseases. These disease include HSV-1 keratitis (Martin et al. 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 transplantation (Omoto et al. 2012), and corneal abrasion (Li et al. 2011). HSV-1 keratitis and diabetic keratopathy result in diffuse damage to the corneal innervation, however some degree of corneal innervation remains intact providing a potential source for corneal reinnervation if the disease is treated appropriately. Lamellar flap surgery, corneal transplantation and corneal abrasion result in damage to portions of the corneal innervation and can result in corneal hypoesthesia. However, normal corneal nerves remain intact in other parts of the cornea and these nerves again provide a potential source of corneal reinnervation from the native corneal innervation.

These above clinical scenarios are very different from NK in the context of complete corneal denervation, in which case there exists no source of corneal reinnervation from the native corneal nerves. All the animal models investigate regeneration of the native corneal innervation, and therefore none of the models ablate the native corneal innervation for a sufficient period to permit the investigation of corneal nerve regeneration from a donor nerve source, as is the case with corneal neurotization. Using these models would permit regeneration of the native corneal innervation in addition to reinnervation from the donor nerve, making it impossible to attribute
changes in the corneal epithelium to reinnervation of the cornea from the donor nerve. Investigation of corneal neurotization requires a novel animal model of NK, in which there is no reinnervation of the cornea by the native corneal innervation, and a novel surgical model of corneal neurotization.
1.7 Thesis Aims and Hypothesis

The primary aims of this thesis are to investigate the impact of corneal neurotization (i.e. corneal reinnervation from a donor nerve) on corneal sensation, ocular surface health, and corneal epithelial healing after injury through a prospective clinical study and the design of a novel animal model of corneal neurotization. A secondary aim is to investigate whether acetyl-L-carnitine and N-acetyl cysteine administered after peripheral nerve injury, improve sensory and motor neuron survival. Nerve transfers that are used for corneal neurotization necessitate a complete transection injury. Improving neuronal survival after transfer may maximize axon regrowth into the cornea and therefore outcomes after corneal neurotization.

This research impacts the adoption of a novel surgical treatment for NK, which has the potential to greatly improve outcomes and rescue vision in these patients. If effective, corneal neurotization may change the treatment paradigm for these patients.

The experimental work included three discrete studies, with the following specific aims and hypotheses:

**AIM 1:** Investigate corneal reinnervation and clinical outcomes in patients with NK after corneal neurotization, including corneal sensation, visual acuity and ocular surface health (see Chapter 2).

Hypothesis: Following corneal neurotization, the cornea will demonstrate corneal reinnervation and patients will demonstrate improved corneal sensation, visual acuity, and ocular surface health.

**AIM 2:** Develop a rat model of neurotrophic keratopathy in which the corneal remains denervated for four weeks (see Chapter 3).

Hypothesis: Sustained corneal denervation following ablation of the corneal reinnervation and prevention of corneal reinnervation for a minimum of 4 weeks is possible in rats.
**AIM 3:** Develop a rat model of corneal neurotization in rats with neurotrophic keratopathy and investigate whether corneal neurotization improves corneal healing after injury (see Chapter 4).

Hypothesis: Rats receiving corneal neurotization will demonstrate significant increases in corneal nerve density, which will derive from the contralateral trigeminal ganglion. Following injury to the corneal epithelium in a rat model of neurotrophic keratopathy, rats with corneal neurotization will heal significantly more quickly than rats with absent corneal innervation.

**AIM 4:** In a rat model of peripheral nerve injury, investigate whether treatment with N-acetyl cysteine or acetyl-L-carnitine reduce motor and sensory retrograde neuron death after injury (see Chapter 5).

Hypothesis: N-acetyl cysteine and acetyl-L-carnitine will demonstrate improved sensory neuron survival after neonatal peripheral nerve injury and N-acetyl cysteine will demonstrate improved motor neuron survival.
Chapter 2 Surgical Reinnervation of the Cornea in Patients with Neurotrophic Keratopathy

This chapter is modified from the following:

2.1 Abstract

**Background:** Neurotrophic keratopathy (NK) is caused by loss of sensory innervation to the cornea. Patients with NK are susceptible to occult corneal injury and poor corneal healing which causes scarring of the cornea and inevitable vision loss. Surgical reinnervation of the cornea restores corneal sensation and may prevent vision loss in these patients.

**Methods:** In this single-center prospective cohort study, patients with advanced NK and vision loss were treated with surgical corneal reinnervation. Pre- and post-operatively we measured corneal sensation, best spectacle corrected visual acuity (BSCVA), and metrics of ocular surface health. Immunohistochemistry and magnetoencephalography (MEG) were used to document corneal reinnervation.

**Results:** Sixteen patients (19 eyes) with NK underwent surgical reinnervation of the cornea. Median central corneal sensation improved from 0 mm pre-operatively (range, 0 to 10) to 60 mm (range, 0 to 60) post-operatively ($p < 0.001$). With a mean follow-up of 24 months (range, 6 – 53), vision was either stable or improved in 16 eyes. Vision further improved in three eyes that underwent successful corneal transplantation 24 to 33 months after corneal reinnervation to correct preexisting corneal scarring. Corneal reinnervation after surgery was confirmed with immunohistochemical analysis of explanted corneal tissue and magnetoencephalography (MEG).

**Conclusions:** Surgical corneal reinnervation restores corneal sensation, improves ocular surface health and preserves vision in patients with NK. Early surgical corneal reinnervation in NK may change the treatment paradigm for these patients by restoring corneal innervation and preventing the complications of NK.
2.2 Introduction

Corneal sensation protects the eye from injury and the corneal nerves produce trophic mediators necessary to maintain and repair the corneal epithelium (Müller et al. 2003). Patients with impaired corneal sensation develop neurotrophic keratopathy (NK), which is characterized by recurrent breakdown of corneal epithelium and poor healing after injury (Sigelman & Friedenwald 1954; Alper 1975; Cavanagh & Colley 1989; Beuerman & Schimmelpfennig 1980; Baker et al. 1993; Chan & Haschke 1982; Chan & Haschke 1981). Over months to years, scarring from repeated ulcerations causes permanent vision loss (Rosenberg 1984; Ramaesh et al. 2007; Lambley et al. 2014; Yamaguchi, Turhan, Deshea L Harris, et al. 2013; Ferrari et al. 2011). NK can be caused by congenital atrophy of the trigeminal ganglion (CN V), or traumatic, infectious or metabolic injury to any part of the corneal innervation.

Even with optimal ophthalmic management, corneal epithelial breakdown and vision loss in patients with advanced NK is inevitable. Conventional treatments, including topical lubricants, and surgical tarsorrhaphy (partial fusion of the eyelid margins) fail to prevent vision loss because they do not address the lack of corneal innervation (Sacchetti & Lambiase 2014; Goins 2005). Corneal transplantation in patients with NK is contraindicated for visual rehabilitation because the donor grafts heal poorly and rapidly opacify in the absence of corneal innervation (Lambley et al. 2014).

Nerve grafts and transfers are an established surgical method to restore motor and sensory function in patients with facial paralysis and traumatic nerve injuries of the upper or lower limb (Terzis & Konofaos 2008; Lee et al. 2008). The success in treating these other conditions provides a strong rationale for investigating nerve grafts and transfers to restore corneal innervation in patients with NK. Surgical reinnervation of the cornea (i.e. corneal neurotization) can be performed by guiding regenerating axons from functioning donor nerves in the face into the cornea through nerve grafts that are transplanted into the affected cornea (Elbaz et al. 2014; Bains et al. 2015). Surgical reinnervation of the cornea may restore corneal sensation and nerve-derived trophic support, thereby preventing vision loss of vision in patients with NK. The objectives of the current study were to demonstrate corneal reinnervation after corneal neurotization and to assess the effect of corneal neurotization on ocular surface health and visual acuity.
2.3 Materials and Methods

2.3.1 Study Design and Oversight

This prospective cohort study was conducted at The Hospital for Sick Children (SickKids), University of Toronto, Toronto, Ontario, and was approved by the SickKids Research Ethics Board. All patients provided informed written consent. The study was performed in keeping with the declaration of Helsinki for research involving human subjects. We measured the efficacy of corneal neurotization in a consecutive cohort of pediatric and adult patients with neurotrophic keratopathy (NK) who had failed conventional ophthalmic therapies, as demonstrated by corneal scarring and decreased visual acuity. The primary end points were corneal sensation and best spectacle corrected visual acuity (BSCVA). Corneal reinnervation after neurotization was assessed using immunohistochemistry and MRI / magnetoencephalography (MEG).

2.3.2 Patients and Interventions

All patients with clinically significant NK and lacking protective corneal sensation (Cobo et al. 1987) were eligible. The study included a consecutive cohort of patients and no patient was excluded from the study. The majority of patients treated were pediatric, however the patient population included two adult patients referred for corneal neurotization. The patient details are summarized in Table 1. Corneal neurotization was performed using a sural nerve graft coapted to functioning sensory nerves elsewhere on the face, most commonly the supratrochlear nerve (Bains et al. 2015). We also report the outcomes of three patients that underwent corneal transplantation after corneal neurotization in an effort to restore corneal clarity and improve vision.

2.3.3 Clinical Assessments

Preoperatively, patients underwent a comprehensive baseline ophthalmic examination, including best spectacle corrected visual acuity (BSCVA), corneal sensation testing, slit-lamp
biomicroscopy and dilated fundoscopy. Corneal sensation testing was performed prior to the use of any eye drops. The level of sensation was measured in the center of the cornea with Cochet-Bonnet esthesiometry (Luneau Ophthalmologie, Chartres, France) (Elbaz et al. 2014). Greater than or equal to 20 mm represents a protective level of corneal sensation (Cobo et al. 1987), while 60 mm corresponds to the maximum length tested with Cochet-Bonnet esthesiometry and 60 mm of corneal sensation being considered indistinguishable from that of unaffected corneas with normal corneal sensation. Prior to corneal neurotization, a systematic facial sensory examination was performed using facial Semmes-Weinstein monofilaments to document which facial sensory nerves were available for use as a donor nerves for corneal neurotization.

Patients were assessed one week and one month after surgery, and then every three months or as needed, afterwards. At each time point, BSCVA, corneal surface integrity, episodes of persistent epithelial defect (PED, defined as non-infected corneal ulceration that failed to resolve within two weeks of commencing ophthalmic treatment) (Lange et al. 2009), Cochet-Bonnet corneal sensory measurement, adjunctive treatments and any side effects or complications from corneal neurotization surgery were documented.

2.3.4 In-vivo Corneal Confocal Microscopy

Two patients underwent in-vivo corneal confocal microscopy (IVCCM) postoperatively to document the presence of sub-basal corneal nerves. Each patient underwent bilateral investigation using a 0.3 – mm² field-of-view lens on the Rostock Cornea Module of the Heidelberg Tomograph II (Heidelberg Engineering, Smithfield, RI) under topical anesthesia. Volume-scanning mode was used to capture a contiguous set of 40 images in the central and peripheral quadrants of the cornea.

2.3.5 Magnetoencephalography

Magnetoencephalography (MEG) recordings were conducted in a 34 year old patient both prior to and 8 months after corneal neurotization. Her contralateral supratrochlear nerve served as the donor sensory nerve. The left and right cornea were stimulated using air-puffs adjusted to the
patient’s blink reflex threshold (0.6 pulses/second for 6 minutes). Tactile stimuli were also applied to the supratrochlear region of the left and right forehead using an inflating plastic membrane taped to the skin surface (approx. 200 stimuli at 0.8 pulses/second) to assess sensation to the forehead. Source localization was carried out on the stimulus-locked averaged evoked responses (1 to 30 Hz) at 50 millisecond latency (Nevalainen et al. 2006) using an event-related beam-forming algorithm (Cheyne et al. 2007) and superimposed on the patient’s structural (T1) magnetic resonance imaging (MRI).

2.3.6  Histology and Immunohistochemistry

Histological and immunohistochemical analyses were performed on formalin-fixed and paraffin-embedded corneal specimens from three patients undergoing corneal transplantation after neurotization. Corneal specimens were fixed in 10% neutral buffered formalin and then bisected twice to produce four crescent-shaped corneal specimens. Corneal sections were cut at 3 microns and examined using hematoxylin and eosin (H&E), trichrome, periodic acid-Schiff (PAS) and iron stains. Nerves were identified with neurofilament antibody clone 2F11 (Dako, Canada) using the DAKO Omnis platform. A formalin-fixed surgical specimen of the cerebral cortex served as a positive control. Comparison was made to samples from six normal corneas from a donor eye bank. In one patient, additional comparison was made to corneal tissue explanted from a corneal transplant prior to neurotization.

2.3.7  Statistical Analysis

All patients who received corneal neurotization were included in the efficacy and safety analysis. Data are reported as of March 1, 2017 and presented as means +/- standard deviations (SD). Pre- and post-operative central corneal sensation and BSCVA (in LogMAR conversion) were calculated and compared via Wilcoxon signed-rank test. Categorical data were compared using Fisher’s Exact Test. A p value < 0.05 was considered to be statistically significant.
2.4 Results

2.4.1 Patient Characteristics

Sixteen patients with corneal hypoesthesia (median 0 mm; range 0 – 10 mm) and NK were enrolled into the study between November 2012 and November 2017. Three patients had bilateral corneal hypoesthesia and neurotrophic keratopathy (NK), resulting in a total of 19 eyes treated with corneal neurotization. Fourteen pediatric patients and two adult patients were included in this study. The etiology of NK was a congenital lack of corneal innervation in thirteen eyes (68 %), traumatic injury in five eyes (26 %) and herpes simplex viral-keratitis in one eye (6 %). In the two adult patients, aged 34 and 22, the etiology of NK was traumatic and congenital, respectively. All but two eyes had a history of persistent epithelial defects (PED) and all eyes had significant corneal scarring interfering with vision. Baseline patient characteristics are summarized in Table 2-1.

Treatments prior to corneal neurotization failed to prevent disease progression and included topical lubricants (84 %), autologous serum-derived eye drops (11 %), and surgical tarsorrhaphy (58 %). Tarsorrhaphy is a partial or complete surgical closure of the eyelids in order to protect the cornea from injury. However, tarsorrhaphy is disfiguring and can block peripheral vision. One pediatric patient underwent fine-needle corneal diathermy and subconjunctival bevacizumab injection, and one other patient received a custom protective scleral contact lens (PROSE lens) (Agranat et al. 2016) to try to slow the progression of vision loss. Four eyes (21 %) had previously undergone emergency corneal transplantation to restore structural integrity of the cornea because of corneal perforation. All of these corneal grafts quickly developed extensive NK-associated corneal scarring resulting in significant vision loss.
Table 2–1 Baseline Patient Characteristics and Outcome

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Pre-Operative (n = 19)</th>
<th>Post-operative (n = 19)</th>
<th>Range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (± STD) or Median (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>Mean</td>
<td>11.9 (± 8.5)</td>
<td></td>
<td>1.9 to 34.3</td>
</tr>
<tr>
<td>Gender (eyes)</td>
<td>Male</td>
<td>8 (42 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11 (58 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etiology (eyes)</td>
<td>Congenital</td>
<td>13 (68 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Traumatic</td>
<td>5 (26 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSV-keratitis</td>
<td>1 (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK Duration (yrs)</td>
<td>Mean</td>
<td>6.0 (± 6.2)</td>
<td></td>
<td>0.5 to 22.5</td>
</tr>
<tr>
<td>Affected Cornea (eyes)</td>
<td>Left (Unilateral)</td>
<td>7 (37 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Right (Unilateral)</td>
<td>6 (32%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bilateral</td>
<td>6 (32 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual Co-morbidities (eyes)</td>
<td>Amblyopia</td>
<td>5 (26 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Facial nerve palsy</td>
<td>5 (26 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cataract</td>
<td>2 (11 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up (months)</td>
<td>Mean</td>
<td>24 (± 16.1)</td>
<td></td>
<td>6 to 53</td>
</tr>
</tbody>
</table>

Outcomes Measures

|                         | Mean (± STD) or Median (95% CI) |       |       |
| CCS (mm) *             | Mean                   | 0.8 (± 2.5)            | 49.4 (± 16.08) | < 0.0001 |
|                         | Median                 | 0 (0 to 0)             | 60 (40 to 60)  | < 0.0001 |
| BCVSA (logMar) **      | Mean                   | 1.03 (± 0.67)          | 0.95 (± 0.75)  | 0.5332   |
|                         | Median                 | 0.7 (0.7 to 1.35)      | 0.6 (0.59 to 1.31) | 0.1992   |
| BCVSA (Snellen)        | Median                 | 20/100 (20/60 to 20/400) | 20/80 (20/40 to 20/800) |       |
| PED (eyes) §           | Total                  | 17 (89 %)              | 4 (21 %)       | < 0.0001 |
|                         | < 1 year of OR         | 10 (53 %)              | 4 (21 %)       | 0.0911 |
| NK Grade (eyes) ¶      | Grade I                | 3 (16 %)               | 15 (79 %)      |       |
|                         | Grade II               | 7 (37 %)               | 4 (21 %)       |       |
|                         | Grade III              | 9 (47 %)               | 0              |       |

*CCS (Central Corneal Sensation); only final follow-up values are shown
**BSCVA; Best Spectacle Corrected Visual Acuity was assessed as LogMAR conversion for statistical purposes. Relative Snellen Conversions are: 20/20 (0 logMAR), 20/200 (1 LogMAR), Light Perception (2.7 logMAR)

§ Persistent epithelial defect (PED); incidence was analyzed both as the total number of patients presenting with a PED prior to neurotization, and those presenting with PED within 1 year prior to surgery and 1 year after surgery

¶ NK Grade; determined using the Mackie Classification

2.4.2 Surgical Details

The surgical details of patients undergoing corneal neurotization are listed in Table 2-2. The corneal neurotization was performed using an autologous sural nerve graft in all patients. Dissection of the graft resulted in a variable number of identifiable fascicles (3.5 ± 1.2) (mean ± SD), which could be dissected and independently implanted into the cornea. As most patients had hemifacial anesthesia, the donor sensory nerve of choice was the contralateral supratrochlear nerve. However, the contralateral supratrochlear nerve was unavailable in a minority of patients, all pediatric, due to sensory abnormalities on pre-operative sensory testing or because the nerve was found to be of insufficient caliber upon intraoperative dissection. In these cases, other facial sensory nerves were used including either the ipsilateral, contralateral supratrochlear, supraorbital or infraorbital nerves.

In the first five patients, four of which were pediatric, the sural nerve was coapted initially in an end-to-side fashion to the donor sensory nerve by making an incision though ~30 % of the nerve diameter. The donor site morbidity was found to be minimal when using this technique, and, in an effort to encourage the regeneration of a higher number of axons into the sural nerve graft to improve corneal sensory outcomes, the sural nerve graft was coapted in an end-to-end fashion in patients 6 through 16.

In the first six patients, the cornea was neurotized by suturing the distal sural nerve fascicles to the sclera at the junction of the scleral-limbal junction. The limbus may present a barrier to axon growth into the cornea. For this reason, patients 7 through 16 were neurotized by placing the sural nerve fascicle directly into the cornea through a stromal pocket.
2.4.3 Clinical Response: Corneal Sensation, Visual Acuity, and Ocular Surface Health

Central corneal sensation (CCS). CCS as measured by Cochet-Bonnet esthesiometry, pre-operatively and then at each post-operative visit after corneal neurotization is demonstrated in Figure 2-1A. Follow-up was variable for each patient because the cohort included patients receiving corneal neurotization over a five-year period from November 2012 to November 2017. Pre-operative CCS was compared to post-operative CCS at 6 months and at the patients final follow-up (Figure 2-1B). CCS significantly improved six months after corneal neurotization, from a median CCS of 0 mm pre-operatively (range, CCS 0 – 5 mm) to 10 mm post-operatively (range, CCS 0 – 60 mm; p < 0.001). By final follow-up (range, 6 to 53 months), median CCS further improved to 60 mm (range, 10 – 60 mm; p < 0.0001) (Figure 2-1B). Average follow-up for the patient cohort was 24 months (range, 6 – 53 months). One eye failed to recover corneal sensation (of > 20 mm) by final follow-up. Reliable testing of corneal sensation was not possible in one patient due to young age and developmental delay.

### Table 2-2 Surgical Details of Corneal Neurotization.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Side</th>
<th># Fascicles</th>
<th>Donor nerve</th>
<th>Coaptation</th>
<th>Stromal Pocket</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L</td>
<td>4</td>
<td>Contralateral supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>7</td>
<td>Contralateral supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>L</td>
<td>4</td>
<td>Ipsilaterial supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2</td>
<td>Ipsilaterial supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>5</td>
<td>Contralateral supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>2</td>
<td>Contralateral supratrochlear</td>
<td>end-to-side</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>L</td>
<td>4</td>
<td>Contralateral supratrochlear</td>
<td>end-to-end</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
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Figure 2–1 Central Corneal Sensation After Corneal Neurotization. (A) Each marker represents the recording for one patient at that follow-up and the average corneal sensation and error bars represent the mean and standard deviation of the Cochet-Bonnet esthesiometry in all the patients at that time point. After corneal neurotization, patients with corneal anesthesia and hypoesthesia began demonstrating improvements in central corneal sensation (CCS), as measured by Cochet-Bonnet esthesiometry, in comparison to pre-operative CCS. (B) The time to CCS improvement was variable between patients. At 6 months after corneal neurotization (blue squares), median CCS improved from a median CCS of 0 mm pre-operatively (range, CCS 0 – 5 mm) to 10 mm post-operatively (range, CCS 0 –
60 mm; \( p < 0.001 \). By final follow-up (range, 6 to 53 months), median CCS further improved to 60 mm (range, 10 – 60 mm; \( p < 0.0001 \)). Average follow-up for the patient cohort was 24 months (range, 6 – 53 months). In patients with longer follow-up, CCS continued to improve up to 24 months, although some patients achieved maximum CCS (60 mm) within six months of corneal neurotization. By final follow-up, all but one patient demonstrated at least protective corneal sensation (i.e. \( \geq 20 \) mm) in the central cornea, while the majority of patients had corneal sensation of 60 mm, which is the maximum score achievable. The dashed line indicates protective corneal sensation (20 mm) and maximum corneal sensation tested (60 mm) is represented with a solid line. Corneal sensation of 60 mm the maximum sensation tested and is considered indistinguishable from normal.

**Best spectacle corrected visual acuity (BSCVA).** BCSVA prior to corneal neurotization and after corneal neurotization at final follow-up is compared in Table 2-1. At final follow-up, 16 of 19 eyes had equal or better vision than before corneal neurotization. Mean BSCVA was not significantly different prior to corneal neurotization (1.03 ± 0.67 LogMAR) and at final post-operative visit (0.95 ± 0.75 LogMAR, \( p = 0.5332 \)). All three eyes that that demonstrated decreased BSCVA after corneal neurotization were in pediatric patients: one was in an eye that had progressive NK after failure of corneal neurotization to improve corneal sensation; one eye had developed a severe cataract impeding vision; and one had dense amblyopia resulting in fluctuations in measured visual acuity. No eyes lost vision due to surgical complications of corneal neurotization.

In order to address dense corneal scarring and improve vision, three eyes underwent corneal transplantation for visual rehabilitation between 24 and 33 months after corneal neurotization. In the three patients that underwent corneal transplantation, central corneal sensation (CCS) decreased post-operatively, but recovered within 6 months in all 3 eyes (range, 40 to 60 mm). The corneal grafts healed well in all patients, suggesting improved ocular surface health after corneal neurotization. In the three eyes that received corneal transplantation, two patients had significant visual improvement after successful corneal transplantation with BCSVA improving from 0.8 LogMar (20/125) to 0.2 (20/30) in one patient and from 2.7 (Light Perception) to 1.78 (20/1200) in the second patient, representing significant improvements in vision. In the third patient, while the cornea remained clear, improvement in visual acuity was limited by pre-existing amblyopia and therefore BCSVA remained unchanged after corneal transplantation. One
patient with improved vision after corneal transplant developed immunologic corneal graft rejection followed by infective ulceration, which resolved and the patient still demonstrated improvement in visual acuity.

**Ocular surface health.** Ocular surface health also improved after corneal neurotization. Preoperatively, 17 eyes (89%) experienced a PED prior to corneal neurotization (Table 2-1). In comparison, during the entire duration of post-operative follow-up (mean 24 ± 16.1 months), only four eyes (21%) experienced PED, representing a significant improvement in ocular surface integrity ($p < 0.0001$). Three of the four patients in whom a PED was diagnosed postoperatively, experienced a single episode that resolved within four weeks with standard medical therapy. Importantly, these three patients were able to feel a change in their ocular surface and therefore self-presented for evaluation earlier than they would have prior to corneal neurotization with absent corneal sensation.

Due to improvements in ocular surface integrity, three eyes (16%) underwent reversal of their tarsorrhaphy after corneal sensation was re-established, restoring normal eyelid opening. Tarsorrhaphy protects the cornea but at the cost of blocking peripheral vision and disfigurement; reversal of tarsorrhaphy therefore improves quality of life. No patient required long-term bandage contact lens or scleral contact lens to maintain ocular surface integrity. Thirteen of 19 eyes (68%) also required fewer topical lubricants to maintain ocular surface integrity and three eyes (16%) continued to receive autologous serum eye drops in the post-operative period.

### 2.4.4 Evidence of Corneal Reinnervation after Corneal Neurotization

Histological examination of the control corneal samples from donors without NK demonstrated linear dot-like profiles positive for neurofilament (NF) antibody in the basal layer of the corneal epithelium and anterior third of the corneal stroma (Figure 2-2).

One patient underwent corneal transplantation prior to corneal neurotization, and so this non-neurotized insensate cornea was available for analysis. The insensate cornea exhibited extensive scarring, chronic inflammation and fibrovascular ingrowth with hemosiderin-laden macrophages. The stroma was thinned and some pigmented iris epithelial cells were found embedded within the fibrous tissue adherent to the posterior surface, consistent with a prior corneal perforation.
Importantly, the corneal sample lacked evidence of innervation on NF staining, with NF immunoreactivity limited to a single axonal profile, therefore consistent with the clinical suspicion of absent corneal innervation and sensation (Figure 2-2).

In contrast, the corneal sample from the same eye one year after neurotization demonstrated uniform thickness of the corneal epithelium with foci of subepithelial fibrosis. The corneal stroma demonstrated easily found thin linear neurofilament immunoreactive profiles (Figure 2-2), indicative of corneal reinnervation after neurotization. Histological examination of the other 2 corneal samples retrieved from corneal transplantation after corneal neurotization also found abundant NF-positive axons. These axons preferentially clustered in the stromal layer and occurred as larger nerve bundles in comparison to normal controls.
**Figure 2–2 Axon Profiles Detectable After Corneal Neurotization.** In normal controls, staining with neurofilament antibody of the corneal sections demonstrated dot and linear axon profiles (arrows) in the subbasal (1A and D) and stromal (1B and C) layers consistent with the normal subbasal and stromal corneal innervation. In contrast, corneal tissue obtained from one patient prior to corneal neurotization demonstrated no subbasal innervation and only a single linear profile with neurofilament immunoreactivity (2B inset) in the stroma, consistent with this patient’s corneal anesthesia. This cornea (2A) also demonstrates a traumatic scar associated with chronic inflammation consistent with neurotrophic keratopathy (NK). 2A inset shows Perl’s iron stain demonstrating hemosiderin containing macrophages within the scar (blue). Macrophages (2B star) were easily recognized and distinguished from neurofilament staining by their globoid shape and light yellow-brown, granular cytoplasm. In the same patient, a corneal transplant specimen two years after corneal neurotization, demonstrated subepithelial discrete clusters of dot and linear profiles (3B) and linear profiles within the stroma (3C), confirming reinnervation of the cornea after neurotization. 3A demonstrates the hematoxylin and eosin staining of the corneal cross-section. These findings are consistent with the patient’s sensory testing, which demonstrated corneal anesthesia prior to surgery (0 mm) and corneal sensation indistinguishable from normal (60 mm) after corneal neurotization.

One patient underwent *in-vivo* corneal confocal microscopy (IVCCM) prior to corneal neurotization and then 6 months after corneal neurotization. Six months after corneal neurotization, thin sub-basal corneal nerves were identifiable in the cornea (Figure 2-3A). This contrasts with imaging prior to corneal neurotization, where the cornea with NK demonstrated significant vascular ingrowth into the cornea with a complete absence of identifiable sub-basal nerve fibers (Figure 2-3C). The normal pattern of sub-basal nerves in the cornea is demonstrated in the same patients unaffected cornea (Figure 2-3D). We were also able to detect sub-basal nerve fibers in a second patient image after corneal neurotization, although this patient had not undergone pre-operative imaging (Figure 2-3B).
In vivo corneal confocal microscopy (IVCCM) was used to identify sub-basal nerve fiber profiles in the cornea in two patients six months (A) and two years (B) after corneal neurotization (identified by yellow arrows). In contrast, IVCCM was obtained in the same patient shown in Panel A prior to corneal neurotization, and no nerve fibers were found in the cornea affected with NK. In contrast, obvious corneal neovascularization (identified by red arrows) were visualized in the cornea after neurotization. Neovascularization of the cornea is a known complication in patients with NK, and decreases corneal clarity. The normal appearance of corneal nerves in Bowman’s Layer is illustrated in the patient’s contralateral (unaffected) cornea (Panel D).
In one adult patient, pre-operative MRI/MEG identified an absence of evoked response in the insensate cornea upon stimulation, consistent with the clinical diagnosis of corneal anesthesia and neurotrophic keratopathy (Figure 2-4, top row). Eight months after corneal neurotization, repeat MEG demonstrated an evoked response localized to the ipsilateral (right) somatosensory cortex upon corneal stimulation with the same air-puff stimulus (Figure 2-4, second row). With normal corneal sensation, the signal would be expected to localize to the contralateral somatosensory cortex (Moulton et al. 2012). The location of the corneal response after neurotization also corresponded to the same cortical region supplying the contralateral forehead (Figure 2-4, third and fourth rows), which is supplied by the supratrochlear nerve that was used to reinnervate the cornea. MRI/MEG therefore demonstrated that the corneal sensation after neurotization was derived from the donor supratrochlear nerve and the corresponding region of the somatosensory cortex.
Figure 2–4 Magnetoencephalography (MEG) Demonstrates Corneal Reinnervation. The donor left forehead dermatome exhibited sensory responses to forehead stimulation (i.e. somatosensory evoked fields [SEFs]) both pre- and post- corneal neurotization. Evoked potentials coincided with the time of stimulation (identified by black arrows), confirming intact sensation to the left forehead. Prior to corneal neurotization, stimulation of the anesthetic right cornea produced no discernable sensory response (SEF), consistent with absent corneal sensation prior to corneal neurotization (right, top panel). Eight months after corneal neurotization, corneal stimulation demonstrated clearly discernable SEFs (identified by black arrows), confirming restored corneal sensation post-neurotization. When the localization of the SEF was superimposed on the patient’s T1 MRI, the corneal signal post-neurotization was localized to the ipsilateral somatosensory cortex (yellow arrow). This situation contrasts with the normal innervation pattern, in which SEFs would ordinarily localize to the contralateral somatosensory cortex. These findings
are consistent with the patient’s surgical procedure in which the donor contralateral supratrochlear nerve reinnervated the anesthetic cornea. Technical details: The averaged somatosensory evoked field (SEF) responses of the MEG sensors overlying the right sensory cortex are shown. Each were time-locked to the sensory stimulus onset for air puff (time-zero) of the right cornea (right top rows) and mechanical stimulation of the left forehead (right bottom rows). A black arrow below the SEF time-course indicates the time corresponding to each stimulus pulse. The left panel demonstrates the source localization of the P50 component (an expected somatosensory response biomarker occurring 50 ms following a stimulus) (Hämäläinen et al. 1990) of SEFs superimposed on the patient’s MRI (80% threshold).

2.4.5 Safety

The surgical procedure was well tolerated by all patients. No patients experienced infections or complications in the immediate post-operative period. Initially, nerve grafts were sutured to the scleral surface with 9-0 Nylon suture. After the first 5 patients we changed to using 9-0 absorbable Vicryl sutures since the Nylon sutures were causing conjunctival irritation and subsequently became exposed.
2.5 Discussion

Corneal anesthesia causes severe neurotrophic keratopathy (NK), which is characterized by persistent corneal epithelial breakdown, extensive scarring of the cornea and permanently impaired vision (Ramaesh et al. 2007; Lambley et al. 2014). Without sensation, the cornea is susceptible to injury and lacks nerve-derived trophic mediators that are necessary for maintenance and repair of the corneal epithelium (Müller et al. 2003; Ueno et al. 2012). NK remains one of the most difficult ocular disease to treat, and conventional treatments for NK fail to prevent blindness in many patients (Sacchetti & Lambiase 2014).

Corneal neurotization corrects the underlying pathophysiology of NK by reinnervating the cornea with functioning sensory nerves from elsewhere on the face. Other strategies to reinnervate the cornea have not been adopted to treat patients due to their more invasive nature, requiring either a fronto-orbital craniotomy or bicoronal incision and extensive dissection (Samii 1981; Terzis et al. 2009). We have minimized the morbidity required to reinnervate the cornea by using a nerve graft and two small supraorbital incisions to guide regenerating axons into the cornea (Elbaz et al. 2014; Bains et al. 2015). Using Cochet-Bonnet esthesiometry, MEG, and ex vivo immunohistochemistry, we are the first to demonstrate that corneal neurotization reinnervates the cornea and restores corneal sensation. Furthermore, patients treated with corneal neurotization experienced no further decline in vision and fewer persistent epithelial defects despite de-escalation of other conventional treatments, including fewer lubricants and reversal of tarsorrhaphy. These results suggest that axons reinnervating the cornea also restore nerve-derived trophic support to the corneal epithelium.

Three patients with NK also underwent corneal transplantation after corneal neurotization. In two patients, transplants remained clear without any complications at 14 and 19 months after corneal surgery, further suggesting that corneal reinnervation restores nerve-derived trophic support and improves corneal healing. The third patient had a clear uncomplicated corneal graft for 10 months prior to experiencing immunologic rejection unrelated to NK. Once the episode of corneal graft rejection resolved, the patient demonstrated improved visual acuity. These results differ significantly from the success of corneal transplantation in this same population prior to corneal neurotization, in which corneal grafts in four eyes for structural repair of the cornea after injury quickly became scarred and opacified resulting in no improvement in visual acuity.
Enabling corneal transplant is an important milestone in the treatment of NK, since previously such transplants to improve corneal clarity were contraindicated for visual rehabilitation because they would fail due to lack of protective sensation and poor healing of the corneal epithelium. We anticipate that this approach will enable restoration of vision in the many thousands of patients currently affected by vision loss in NK.

There are limitations to this study. As a prospective cohort study, our patients all received standard ophthalmic care in addition to corneal neurotization. We were therefore unable to compare corneal neurotization to standard management in a controlled manner. Our study included a young cohort and results may not be applicable to older patients. The most common etiology of NK was congenital or traumatic, thus the effectiveness of neurotization is perhaps less certain in patients with infectious or metabolic corneal nerve injury.

Our study demonstrates that corneal neurotization reinnervates the cornea, improves ocular surface health and maintains visual acuity in patients with NK. Corneal neurotization also improves the success of corneal transplantation, providing an opportunity for further visual rehabilitation in patients with NK. Surgical reinnervation represents a significant change in the treatment paradigm for NK, and our results justify investigating corneal neurotization as a first-line treatment to prevent complications in patients with NK.

While NK is a rare disorder, affecting approximately 5 in 10,000 people, a much larger population of patients exists worldwide with corneal nerve dysfunction as a result of diabetes, herpes simplex keratitis or leprosy. Further studies are required to determine whether increasing corneal innervation with corneal neurotization is effective in these patients. If effective, the impact on global health would be quite significant as these are significant causes of corneal blindness worldwide.
Chapter 3 Development and Validation of a Rat Model of Neurotrophic Keratopathy
3.1 Abstract

**Background:** Loss of sensory innervation to the cornea causes neurotrophic keratopathy (NK), which is characterized by corneal scarring and vision loss due to breakdown and poor healing of the corneal epithelium. Corneal neurotization is a novel surgical procedure that uses foreign donor nerves from elsewhere on the face to reinnervate the cornea in patients with NK. Clinical studies have demonstrated that following corneal neurotization, donor nerves reinnervate the cornea and restore corneal sensation. However, it is not known whether the donor axons that reinnervate the cornea after corneal neurotization contain the neuromediators that are essential to prevent breakdown of the corneal epithelium. Development of an animal model of NK and corneal neurotization is required to address these issues and, in turn, to understand whether corneal reinnervation by a foreign donor nerve prevents vision loss in patients with NK.

**Methods:** A literature review was performed to identify the animal models of NK that cause complete denervation of the cornea. Two methods were selected for their effectiveness and reproducibility: i) transection of the long ciliary nerves, and, ii) stereotactic electrocautery of the ophthalmic nerve. These methods were used in the *thy1*-GFP+ rat, which expresses green fluorescent protein (GFP) in all axons, permitting visualization of corneal denervation after injury to the native innervation. Cadaveric dissections were performed to determine the correct stereotactic coordinates for corneal denervation. Electrosurgical variables pertaining to stereotactic electrocautery of the ophthalmic nerve (V1) and trigeminal ganglion (TG), including electrosurgical power, mode and duration, were optimized in a series of rats to minimize rat morbidity after the electrocautery. Corneal denervation and reinnervation after stereotactic electrocautery and long ciliary nerve transection was assessed at one, two, and four weeks after corneal nerve injury with corneal harvest and whole mount microscopy.

**Results:** The lateral conjunctival approach to transect the long ciliary nerves was effective in completely denervating the cornea. However, abundant axon regrowth was apparent at two and four weeks. Attempts to decrease axon regeneration with topical ethanol proved to be ineffective and it also damaged the vascular supply to the ocular surface. Stereotactic electrocautery of the TG was poorly tolerated by the rats, resulting in no animal surviving to four weeks. In contrast, stereotactic electrocautery of the ophthalmic nerve was well tolerated. Electrosurgical settings of 10 W for 60 seconds on Cut/Pure mode resulted in the most consistent corneal denervation.
without compromising rat survival. Some axon reinnervation of the central cornea was present four weeks after stereotactic electrocautery of V1. In order to ensure the cornea was maximally denervated at four weeks after the initial V1 electrocautery procedure, V1 electrocautery was performed a second time 3 weeks after the first procedure.

**Conclusions:** V1 electrocautery is well tolerated in rats and results in complete corneal denervation. However, as some corneal reinnervation frequently occurs by four weeks after injury, a second V1 electrocautery procedure should be performed three weeks after the first to ensure that the native corneal innervation is completely ablated for the entire four week period.
3.2 Introduction

Corneal sensation is necessary to protect the eye from injury and the corneal nerves produce neuromediators essential for corneal epithelial maintenance and repair (Müller et al. 2003). Patients with impaired corneal innervation develop neurotrophic keratopathy (NK), characterized by recurrent and persistent corneal epithelial breakdown, that leads to significant corneal scarring and progressive, permanent vision loss (Rosenberg 1984; Ramaesh et al. 2007; Lambley et al. 2014; Yamaguchi, Turhan, Deshea L Harris, et al. 2013; Ferrari et al. 2011). Conventional treatments, including lubricating eye drops and surgical tarsorrhaphy (sewing the eyelids together), protect the corneal surface from injury but fail to address the underlying cause of NK and prevent vision loss in these patients (Sacchetti & Lambiase 2014; Goins 2005).

Corneal neurotization is a novel surgical procedure that reinnervates the cornea using functioning donor sensory nerves from elsewhere on the face. Several studies have demonstrated improved corneal sensation after corneal neurotization (Samii 1981; Terzis et al. 2009; Elbaz et al. 2014). Terzis et al. (2009) also demonstrated improved vision and corneal health in patients treated with corneal neurotization, however all patients were also treated concurrently with conventional ophthalmic management making it impossible to attribute changes in vision or corneal health solely to corneal neurotization. It remains uncertain whether donor nerves that reinnervate the cornea after corneal neurotization contain the neuromediators that are necessary for normal corneal epithelial maintenance and repair, such as Substance P, that are supplied by the normal corneal innervation.

Definitively demonstrating improved vision and ocular surface health in patients with NK requires a large randomized controlled trial (RCT). However, there are several challenges in designing an RCT in patients with NK because NK is a rare condition with an estimated prevalence of 5 in 10,000 people (Sacchetti & Lambiase 2014). This makes the recruitment of patients for a RCT difficult. NK is also a heterogenic disorder and hence, would require a large sample size to detect differences. Due to the poor outcomes of conventional ophthalmic management, there is no clinical equipoise that provides a rationale for restricting access of patients to the surgical procedure of corneal neurotization. Therefore, in addition to larger prospective cohort studies, non-clinical research methods are required to further our understanding of how corneal neurotization influences the corneal epithelium in patients with
NK. An animal model of corneal neurotization would allow for the characterization of nerve fibers that reinnervate the cornea and may be used to determine whether these nerves improve the maintenance and healing of the corneal epithelium.

There have been no previous animal studies investigating corneal neurotization. However, several rodent and rabbit models have been described that investigate regeneration of the native corneal nerves in HSV-1 keratitis (Martin et al. 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 transplantation (Omoto et al. 2012), and corneal abrasion (Li et al. 2011). These models are unsuited for the investigation of corneal reinnervation by a foreign donor nerve, as is the case in corneal neurotization in NK patients, as these animal models do not result in complete denervation of the cornea with consequent robust corneal reinnervation by the native corneal nerves. In an animal model of corneal neurotization, the native corneal innervation must first be completely ablated in order to attribute changes in the cornea to the donor nerve population.

An animal model of corneal neurotization first requires a model of NK in which the corneal innervation is completely ablated. Several animal models of NK have been described in monkeys (Alper 1975), rabbits (Zander & Weddell 1951; Schimmelpfennig & Beuerman 1982) and rodents (Sigelman & Friedenwald 1954; Tervo et al. 1979; Keen et al. 1982; Gallar et al. 1990; Nagano et al. 2003; Ferrari et al. 2011; Yamaguchi, Turhan, Deshea L. Harris, et al. 2013). Both stereotactic surgery to ablate the ophthalmic nerve (V1) (Nagano et al. 2003; Wong et al. 2004; Ferrari et al. 2011) and a lateral conjunctival approach to transect the long ciliary nerves (Yamaguchi, Turhan, Deshea L. Harris, et al. 2013) have demonstrated reliable denervation of the cornea in rodents for one week, but neither study has investigated whether corneal reinnervation from the native corneal innervation occurs subsequently.

Here we describe the development of a rat model of NK in the thy1-GFP+ Sprague-Dawley rat, which expresses green fluorescent protein (GFP) in all axons. This NK model was developed with the intention of providing a minimum of four weeks of corneal denervation so as to investigate corneal neurotization. A four week time period of sustained corneal denervation is hypothesized to be required for donor axons to regenerate and reinnervate the cornea. We investigated three potential methods of corneal denervation: i) stereotactic electrocautery of the
V1, ii) stereotactic electrocautery of the trigeminal ganglion (TG), and iii) transection of the long ciliary nerves.
3.3 Materials and Methods

3.3.1 Study Design

A model of neurotrophic keratopathy (NK) was developed in the Thy1-GFP+ Sprague Dawley (SD) rat. This rat strain expresses green fluorescent protein (GFP) in all axons and permits the visualization of corneal axons without requiring immunohistochemistry (Figure 3-1). A literature review was performed to identify existing techniques to ablate the corneal innervation. Two techniques that were demonstrated to be reliable and reproducible in mice, were selected: i) transection of the long ciliary nerves through a lateral conjunctival incision, and ii) stereotactic electrocautery of the ophthalmic nerve (V1 electrocautery). In addition, we investigated a third technique of stereotactic electrocautery of the trigeminal ganglion (TG), as this would destroy the primary sensory neurons located in the TG and therefore permanently prevent reinnervation. These techniques were modified experimentally in an effort prevent native corneal nerve regeneration and reinnervation for a period of four weeks, which was necessary to permit corneal reinnervation from a subsequent source of neurotization.

Transection of the long ciliary nerves was first investigated as a technique of corneal nerve ablation, and subsequently investigated with the addition of topical ethanol (EtOH) to further damage exposed nerve endings and prevent regeneration of the long ciliary nerves after transection. Topical alcohol damages nerves and has been known to cause NK when used in the past to damage the trigeminal nerve in patients with trigeminal neuralgia (Davies 1970). We then investigated stereotactic electrocautery of the TG and V1 separately. Stereotactic electrocautery included multiple variables that had to be optimized, including the method of ablation, stereotactic coordinates, and the energy parameters, including power, pulse-type and duration.
3.3.2 Animals

Seventy-eight 250 to 300 g female Thy1-GFP+ SD rats were used in these experiments. All rats were maintained in a temperature and humidity controlled environment with a 12:12 h light:dark cycle and received ad lib water and standard rat chow (Purina, Mississauga, ON). Surgical procedures were conducted in an aseptic manner with an operating microscope (Leitz, Willowdale, ON). Rats were sacrificed at study termination under deep anesthesia using intraperitoneal (i.p) Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg, Bimeda-MTC, Cambridge, ON). These experiments were approved by The Hospital for Sick...
Children Laboratory Animal Services (LAS), which adheres to the guidelines of the Canadian Council on Animal Care.

### 3.3.3 Transection of the Long Ciliary Nerves

Transection of the long ciliary nerves was performed using a procedure described previously in mice (Yamaguchi, Turhan, Deshea L. Harris, et al. 2013). All aseptic surgical procedures were performed using inhalational anesthetic (2% Isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ). The periorbital hair was shaved, and the area disinfected with povidone iodine. A curvilinear incision was made in the lateral conjunctiva. Dissection was performed through the periocular fat pad to expose the optic nerve in the posterior orbit. The eye was then rotated nasally to provide better visualization of the optic nerve. All soft tissues surrounding the optic nerve were then transected and dissected posteriorly to transect the long ciliary nerves as proximally as possible. Thereafter, the lateral conjunctival incision was closed with 9-0 Vicryl sutures. Antibiotic ointment (Polysporin, Johnson and Johnson Inc., Marham, Canada) was applied to the eye. A tarsorrhaphy (eyelid closure) was performed with a single 6-0 polypropylene suture in order to protect the cornea from injury. Rats received Metacam (0.3 mL/100 g body weight; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) immediately post-operatively and thereafter if symptoms of pain were evident.

### 3.3.4 Determination of the Stereotactic Coordinates of the Ophthalmic Nerve and Trigeminal Ganglion

Ten rats were used to ascertain the appropriate stereotactic coordinates for V1 and TG. A literature search was performed for articles describing the stereotactic location of V1 and TG, including a stereotaxic atlas (Paxinos & Watson 1997; Ahn et al. 2009; Schneider et al. 1981; Jeon et al. 2012; Nagano et al. 2003; Huhtala 1976). Briefly, using the stereotactic frame, we lowered a 22 G monopolar electrode (UP 3/50, Pajunk GmbH, Germany) through the brain at two sets of coordinates for V1 and TG), based on our literature search. Rats were then perfused and V1 and TG dissected to confirm the location of injury. Thereafter, these coordinates were
modified in different animals through trial and cadaveric dissection to determine the correct coordinates for the female thyroid-GFP+ rat weighing between 250 and 300 g.

3.3.5 Stereotactic Electrocautery of the Trigeminal Ganglion and Ophthalmic Nerve

Rats were anesthetized using inhalational anesthetic (2% Isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ) and the head shaved and disinfected with 10% povidone iodine. The rat was mounted on a stereotactic frame (Harvard Apparatus, Holliston, Massachusetts) (Figure 3-2) and a midline cranial incision was made on the skull with a 15-scalpel blade. Hydrogen peroxide and cotton tipped applicators (AMG Medical, Montreal, Quebec) were used to gently remove the pericranium from the skull. Once the pericranium was removed, bregma (the intersection point of the coronal and sagittal sutures) was identified as a point of reference (with the coordinates AP 0.0 mm, ML 0.0 mm, depth 0.0 mm) (Figure 3-3A). A 1 mm burr hole was made with a dental drill in the skull at the coordinates for either V1 or TG through which a 22 G monopolar electrode (UP 3/50, Pajunk GmbH, Germany) was lowered to the correct depth (Figure 3-3 B/C). The insulated monopolar electrode was modified by removing the insulation from 1 mm at the tip of the electrode and blunting the electrode to prevent it from passing through the middle cranial fossa when lowered (Figure 3-4). The monopolar electrode was then connected to the electrosurgical generator (Force FCTM-8C, Medtronic, USA) (Figure 3-5A/B), in order to produce an electrolytic ablative signal at the bared tip of the monopolar electrode. Prior to the application of the electrocautery signal, a grounding pad was placed over a shaved area of skin on the back (Figure 3-5/C).

The electrosurgical generator outputs electrocautery signals at various powers (volts multiplied by the current), stimulus pulse widths and frequencies, resulting in variable injury patterns. We experimented with each of the monopolar modes (i.e. Cut [Pure], Coagulation [Desiccation], Coagulation [Fulguration]). After finding that the Cut (Pure) mode was better tolerated for electrosurgical ablation, each coordinate for V1 and TG were examined separately in a series of 10 rats per coordinate to examine the maximum tolerable power for each coordinate. Power was initially set to 1 W, and then progressively increased to 2 W, 5 W, 10 W, 20 W to identify the minimum power required to ablate V1 and TG without compromising rat survival.
Following stereotactic electrocautery, corneal denervation was confirmed by an absent blink reflex tested with cold saline and a corneal esthesiometer (Luneau Ophthalmologie, Chartres, France) under light inhalational anesthesia. A positive blink reflex was first demonstrated in the contralateral eye for comparison. The electrode was then removed and the skin sutured. A tarsorrhaphy was performed with 6-0 polypropylene suture to reduce the risk of injury to the cornea and rats were provided with buprenorphine (1 mg/kg; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) for post-operative pain relief. All rats receiving stereotactic electrocautery were also provided with dexamethasone (1mg/kg; Sandoz Canada Inc., Boucherville, Canada) to reduce intracranial swelling post-operatively. Animals were placed on a heated pad for recovery.

**Figure 3–2 Stereotactic Frame and Set-up.** The stereotactic frame (A) is equipped with two manipulator arms that are adjustable 80 mm in the ventral-dorsal, medial-lateral and anterior posterior plane with an absolute lock at 90 ° ventral-dorsal. Vernier scales (accurate to 100 µm) are located on the ear bars and frame. The left manipulator arm is equipped with a modification that permanently holds the dental drill perpendicular to the plane of the frame. The right manipulator arm contains a needle holder that ensures that the needle enters the skull perpendicular to the plane of the cranium. The frame is also equipped to permit the use of inhalational anesthetic once the rat is placed on the bite bar and the ear bars (B) ensuring that the surface of the skull is parallel to the base of the frame.
Figure 3–3 Location of Bregma and Insertion of the Insulated Needle. Once a midline incision was made over the scalp, hydrogen peroxide was used to assist in removing the pericranium from the skull. Once the pericranium was removed, the coronal and sagittal sutures were readily identifiable (A). The intersection of the coronal and sagittal sutures is referred to as bregma, which is the point of reference with the coordinates (AP 0.0 mm, ML 0.0 mm, VD 0.0 mm) from which other stereotactic coordinates are calculated. A dental drill was then used to make a 1 mm burr hole through the skull at the coordinates for either the V1 or the TG (shown in B). The above image demonstrates a burr hole made at the coordinates (AP +1.5 mm, ML + 2.0) that were used to ablate V1. The insulated needle (C) was then slowly lowered through the burr hole to the appropriate coordinate for the V1 and TG.

Figure 3–4 Modification of the SonoPlex STIM Needle. A SonoPlex STIM needle (A) manufactured by Pajunk was modified in order to deliver an electrosurgical signal to the ophthalmic nerve (V1) and trigeminal ganglion (TG) while sparing the rest of the intracranial contents. These needles are insulated with an ultrathin NanoLine coating protecting the brain from electrocautery as the needle passes through it towards the trigeminal nerve. To improve tissue ablation, 1 mm of insulation was removed from the tip in order to expose the entire depth of the V1 or TG to uninsulated needle tip. The tip
of the needle was then also blunted to prevent passage of the needle through the middle cranial fossa and ensure that the needle remained in contact with either the V1 or TG (B/C).

**Figure 3–5 Stereotactic Electrocautery Set-up.** The insulated electrocautery needle was connected from the input on the needle to the output of the electrosurgical generator with a modified banana plug (A). The electrosurgical generator was a Force FX unit supplied by Covidien (B). A return pad was placed on the back of the rat prior to electrosurgical ablation (C).

### 3.3.6 Harvest of the Ophthalmic Nerve and Trigeminal Ganglion

Following corneal harvest, rats were sacrificed at study termination under deep anesthesia using intraperitoneal (i.p) Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg, Bimeda-MTC, Cambridge, ON). Rats were then perfused with 250 mL of cold normal saline followed by 500 mL of 4% paraformaldehyde (PFA). The cranium was dissected and the brain was removed from the cranial cavity in order to expose and visualize the V1 and the TG (Figure 3-6). At this point, visual confirmation was made that the site of injury was in the correct location. The meninges were then carefully lifted off of the ophthalmic nerve and the adjacent bone was carefully removed with a ronguer under visualization with a surgical microscope (Leica M651, Leica Microsystems Inc., Concord, ON).

The trigeminal nerve was then transected distal to the site of injury and proximal to the TG in order to remove the specimen as one continuous block, which was then cryoprotected in 30% sucrose in 4% PFA for four days prior to embedding in optimal cutting temperature compound (OCT: Sakura Fine Technical Co., Torrence, CA). The specimen, containing the V1 and the TG, was serially sectioned at 20 µm using a cryostat (Leica Microsystems Inc., Concord, ON) at -22°C.
C and mounted onto Superfrost slides (Fisher Scientific, Ottawa, ON). Serial sections were examined on a fluorescent microscope with a 10x objective (100x overall magnification; Leica) to examine for the presence of GFP+ signals distal to the site of injury.

Figure 3–6 Harvest of the Trigeminal Ganglion (TG) and Ophthalmic Nerve (V1). After perfusion, the cranium was exposed (A) and then removed with a rongeur to expose the cranial base (B). The ophthalmic nerve (V1, labeled in yellow) is identifiable lateral to the optic nerves, and can be traced proximally to identify the trigeminal ganglion (TG, labeled in red) and the mandibular branch of the trigeminal nerve (V3) (labeled in blue). The meninges and cranial base were then carefully dissected from the V1 and TG in order to removed the nerve and ganglion as one en bloc specimen (C).

3.3.7 Analysis of Trigeminal Injury with Hematoxylin and Eosin

After examining serial sections of the V1 nerve and TG under a fluorescent microscope, additional sections were stained with hematoxylin and eosin staining to visualize the zone of injury after stereotactic electrocautery of V1. Selected slides were fixed in formal alcohol for 30 seconds, followed by washing under running H₂O for 30 seconds. Slides were then stained for 60 seconds in Harris Haemotoxylin solution followed by washing under running water for 20 seconds. Slides were then dyed in ammonia for 20 seconds, washed under running water, and then washed in 95% EtOH for 10 seconds. Slides were then dehydrated in 95% EtOH for 10 seconds and cleared in two changes of Xylene (10 s each).
3.4 Results

3.4.1 Long Ciliary Nerve Transection Results in Significant Corneal Reinnervation After Four Weeks

Ablation of the corneal innervation via transection of the long ciliary nerves resulted in inconsistent denervation of the cornea. Because the long ciliary nerves were difficult to visualize in the \textit{thy}1-GFP+ rat, all the tissue surrounding the optic nerve had to be dissected posteriorly in order to be certain that the long ciliary nerves were transected and the cornea denervated. This did result in more consistent denervation of the cornea as evident by absence of corneal innervation at one week after the long ciliary nerve transection (Figure 3-7A). However, reinnervation of the cornea was visible at two weeks, with significant corneal reinnervation by four weeks after transection (Figure 3-7 B/C).

\textbf{Figure 3–7 Corneal Nerve Regeneration After Long Ciliary Nerve Transection.} Dissection and transection of the entire tissue surrounding the optic nerve behind the orbit was required to produce reliable transection of the long ciliary nerves as demonstrated by complete loss of GFP+ axons in the cornea one week after injury (A). Two weeks after ciliary nerve transection, corneal nerve regeneration was visible as GFP+ axons in the corneal periphery (B). This then progressed to significant corneal nerve regeneration at 4 weeks with extensive reinnervation of the central cornea as well (C). Note that the light intensity was considerably higher in A and B in order to ensure the visibility of any GFP+ axons in the cornea.
3.4.2 Alcohol Application Results in Significant Corneal Damage and Fails to Prevent Corneal Reinnervation at Four Weeks

In order to prevent corneal nerve reinnervation after long ciliary nerve transection, topical alcohol (EtOH) was applied to the transected ciliary nerves to cause further damage and decrease corneal nerve regeneration after ciliary nerve transection. When using 90% EtOH, less reinnervation of the cornea was apparent at two weeks (Figure 3-8A/B), as compared to more reinnervation at this time point without its application (Figure 3-7B). Nonetheless, significant corneal reinnervation was still apparent at four weeks (Figure 3-8C). Less corneal nerve regeneration occurred at four weeks when using 100% EtOH (Figure 4-9C). However the application of alcohol also resulted in concomitant devascularization of the cornea and anterior orbit. The vessels supplying the retina and the anterior orbit also run adjacent to the optic nerve and these appeared to become damaged with topical EtOH. Loss of blood supply to the anterior orbit makes this technique inappropriate for the investigation of corneal reinnervation after corneal neurotization, as a robust blood supply is required to reinnervate the nerve grafts after transfer into the cornea.

Figure 3–8 Regeneration After Long Ciliary Nerve Transection and 90 % EtOH. Alcohol is toxic to nerves, and therefore in an effort to further prevent axon regeneration after long ciliary nerve transection, 90 % EtOH was applied to the posterior orbit after transection of the tissues surrounding the optic nerve. No corneal nerve regeneration was apparent one (A) and two (B) weeks after long ciliary nerve transection. However, four weeks after injury the cornea demonstrated obvious regrowth of GFP+ axons (C).
Figure 3–9 Regeneration After Long Ciliary Nerve Transection and 100 % EtOH. To further damage nerves, absolute alcohol after long ciliary nerve transection was also investigated. The use of absolute alcohol again resulted in no corneal nerve regrowth a 1 (A) and 2 (B) weeks after long ciliary nerve transection. At 4 weeks, the cornea demonstrated fewer GFP+ axons than long ciliary nerve transection alone or 90% EtOH, however there was still significant axon regeneration into the center of the cornea (C). Absolute alcohol also resulted in significant vascular injury to the ocular surface and posterior orbit resulting in revascularization of the retina and cataract formation.

3.4.3 Stereotactic Coordinates for the Ophthalmic Nerve and Trigeminal Ganglion

Initial coordinates for the V1 and TG were determined based on previous literature (Table 3-1). These coordinates were then modified through serial trials and cadaveric dissection to produce the final coordinates used for these studies:

V1: AP + 1.5 mm, ML + 2.0 mm, DV 11 mm

TG: AP – 3.4 mm, ML + 2.4 mm, DV 9.0 mm

These coordinates reliably targeted V1 and TG in the thy1-GFP+ rat. Successful stereotactic electrocautery at each location was confirmed by an absent blink reflex to cold saline and mechanical pressure (Cochet-Bonnet esthesiometry) applied to the cornea post-operatively. The correct location of injury was confirmed upon cadaveric dissection.
Table 3–1 Stereotactic Coordinates for V1 and TG. A literature search and stereotaxic atlas (Paxinos et al., 1997) were used to determine initial stereotaxic coordinates for the trigeminal ganglion and ophthalmic nerve. The final coordinates used in this study were then determined through a series of trials described in the Methods section. AP; anterior-posterior coordinate. ML; medial-lateral coordinate. DV; dorsal-ventral coordinate.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Species (Rat)</th>
<th>Reference</th>
<th>AP</th>
<th>ML</th>
<th>DV</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ahn et al. 2009</td>
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<td>3 mm</td>
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<td>9.6 and 10.7 mm</td>
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<tr>
<td><strong>V1 Branch:</strong></td>
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<td></td>
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<td>Nagano et al. 2003</td>
<td>Brown Norway</td>
<td>Bregma</td>
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<td>Huhtala 1976</td>
<td>SD</td>
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<td>0.5 to 1.0 mm from the base of the skull</td>
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3.4.4 Stereotactic Ablation of the Ophthalmic Nerve and Trigeminal Ganglion Results in Minimal Corneal Reinnervation at Four Weeks

The above coordinates were then used to determine the appropriate electrosurgical mode, power, and duration for stereotaxic intracranial electrocautery of V1 nerve and the TG (Table 3-2 and 3-3). Stereotactic ablation of the TG at the coordinates AP - 3.4 mm, ML + 2.0 mm, DV 9 mm, was poorly tolerated by all rats. The only two rats that survived the procedure to the study endpoint at four weeks were treated with 1 W for 60 seconds. This was insufficient energy to ablate the TG and the corneal innervation. After stereotactic ablation of the TG, some rats awoke from the procedure with ipsilateral left arm weakness that suggested potential damage to the motor cortex or brainstem. All the rats that received TG ablation also demonstrated weakness and poor oral intake that resulted in matting of the fur beneath the jaw. All rats treated with greater than 1
W also demonstrated canting of the jaw to the contralateral side, suggesting paralysis of the muscles of mastication on the side of ablation. This is also consistent with TG ablation because the motor nerve fibers to the muscles of mastication pass through the TG. As a result of these dire consequences, this technique for corneal denervation was abandoned. In two rats that survived to study termination with TG ablation using 3 W of energy, no corneal axon regrowth was visible at one and two weeks, with minimal corneal nerve regeneration at four weeks that was mostly restricted to the peripheral cornea (Figure 3-10).

Table 3–2 Settings for Stereotactic Electrocautery of TG. The electrosurgical generator delivers power in several modes the produce different patterns of injury: Cut (Low/Pure/Blend) and Coagulation (Dessicate/Fulgurate/Spray). Cut mode utilizes lower radiofrequencies at a consistent rate, resulting in heating of tissue and destruction via cellular vaporization while coagulation modes utilize frequencies with interspersed lower frequencies to produce dehydration of tissue and a zone of coagulation injury. Initially, we investigated coagulation modes (Fulgurate/Dessicate) because these modes result in a larger zone of injury however these were poorly tolerated resulting in significant rat mortality and morbidity. Rats were able to tolerate 1 W for 30 or 60 seconds, however this was found to be insufficient to ablate the corneal innervation. Due to poor survival, only 1 rat was used per group in these trials. X, indicates rat sacrificed prior to study end-point due to poor oral intake and weight gain; +, indicates rat reached study end-point and corneal innervation was present when the cornea was imaged; −, indicates rat reached study end-point and corneal innervation was absent when imaged.

<table>
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<tr>
<th>Mode</th>
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<td>Fulgurate</td>
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<td>Dessicate</td>
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<td>3</td>
<td>X</td>
</tr>
<tr>
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<tr>
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<td>3</td>
<td></td>
</tr>
<tr>
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<td>X</td>
</tr>
<tr>
<td>Cut/Pure</td>
<td>5</td>
<td>30</td>
<td>X</td>
</tr>
<tr>
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<td>+</td>
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</tr>
<tr>
<td>Cut/Pure</td>
<td>2</td>
<td>30</td>
<td>X</td>
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Stereotactic ablation of the trigeminal ganglion with electrocautery (Cut/Pure, 3 W, 60 s), resulted in minimal regrowth of corneal axons at 1 (A) and 2 (B) weeks after the procedure. In this example of three rats, there remained little corneal reinnervation four weeks after trigeminal ganglion ablation. This reinnervation was mostly restricted to the corneal periphery (C).

Rats were able to tolerate higher power electrocautery signals at the V1 location while ablation of the TG even at minimal settings resulted in significant postoperative morbidity and mortality in the rats (Table 3-3). Stereotactic electrocautery of the distal V1, at the coordinates AP + 1.5 mm, ML + 2.0 mm, DV 9 mm with 10 W for 60 seconds, resulted in complete corneal denervation at one week, with minimal corneal regeneration restricted to the peripheral cornea at two weeks (Figure 3-11A/B), although there was some reinnervation of the central cornea, four weeks after stereotactic electrocautery (Figure 3-11C). The Cut/Pure mode, which delivers an electrical signal with a 390 kHz sinusoid wave, was found to produce the most specific zone of injury with improved rat survival after the procedure. The signal frequency of this mode is most consistent with two previous studies using radiofrequency thermocoagulation in rats (Nagano et al. 2003; Wong et al. 2004). The procedure was well tolerated in all rats, and no rats required sacrifice prior to the study end-point.

In the rats whose corneas were not protected with a tarsorrhaphy, rats developed the clinical syndrome of neurotrophic keratopathy (NK) with extensive corneal epithelial ulceration, corneal
scarring and eventual corneal perforation in severe cases. These findings confirmed that this method of corneal denervation results in the clinical syndrome of NK.

**Table 3–3 Stereotactic Electrocautery of V1.** Initial parameters were selected based on previous experience with ablation of the trigeminal ganglion. Different powers (watts) and durations of signal (seconds) were investigated using the Cut/Pure mode as this mode was found to be better tolerated when ablating the TG. We started with lower power (2 W) for 30 seconds and then progressively increased the duration and power of signal to determine the maximum tolerated dose of electrocautery with stereotactic surgery. We found that 10 W applied for 60 s was well tolerated and resulted in reliable ablation of the corneal innervation as determined by corneal blink reflex, cadaveric dissection and corneal harvest and confocal microscopy. X, indicates rat sacrificed prior to study end-point due to poor oral intake and weight gain; +, indicates rat reached study end-point and corneal innervation was present when the cornea was imaged; −, indicates rat reached study end-point and corneal innervation was absent when imaged.

<table>
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<th>Mode</th>
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<td>60</td>
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**Figure 3–11 Corneal Nerve Regeneration V1 Electrocautery.** Stereotactic electrocautery of V1 with 10 W for 60 seconds resulted in complete corneal denervation at 1 week (A) with minimal corneal
reinnervation and no GFP+ axons in the central cornea by 2 weeks (B). By 4 weeks there were a small number of GFP+ axons visible throughout the cornea including the central cornea (C).

3.4.5 Stereotactic Ablation of the Ophthalmic Nerve at 0 and 3 Weeks Provides Complete Corneal Denervation at 4 Weeks

While stereotactic electrocautery of ophthalmic nerve (V1) resulted in minimal corneal reinnervation at four weeks, we suspected some nerve regeneration derived from the TG. Therefore we used stereotactic ablation of the V1 nerve branch a second time in the same location, three weeks after the first ablative procedure. The cornea was then harvested four weeks (after the first ablation) to examine for corneal reinnervation. Two procedures of stereotactic ablation noticeably reduced corneal reinnervation four weeks after the initial V1 ablation resulting in minimal reinnervation of the peripheral cornea and no reinnervation of the central cornea (Figure 3-12),

Dissection of V1 demonstrated that the first and second ablative procedures resulted in extensive damage of V1 (Figure 3-13A). After harvest of the ipsilateral and contralateral V1, hematoxylin and eosin staining documented extensive injury to the V1 nerve on the side of stereotactic ablation with few axons remaining in continuity in comparison the contralateral TG (Figure 3-13 B-E). An absence of GFP+ axons distal to the site of injury was also seen with immunofluorescence (Figure 3-13 F/G), suggesting extensive damage to V1 consistent with absent corneal innervation as seen in Figure 3-12.
Figure 3–12 Corneal Reinnervation after Two Stereotactic Electrocautery Procedures of V1. Stereotactic ablation of the ophthalmic nerve V1 using 10 W for 60 seconds (Cut/Pure mode) significantly decreased corneal axon density four weeks after injury (A/B). However, reinnervation of the cornea was variable with some rats demonstrating central corneal reinnervation (A) with other demonstrating less reinnervation (B). Note that the area of hyperdense innervation at the top of B is an artifact from ciliary muscle that was adherent to the cornea and was not corneal nerve reinnervating the cornea. Two procedures of stereotactic electrocautery of V1, with the second procedure occurring three weeks after the first, significantly reduced corneal reinnervation 4 weeks after the first procedure of stereotactic electrocautery with less variability between animals and more consistency of central corneal denervation (C/D).
**Figure 3–13 V1 Injury After Stereotactic Electrocautery.** Dissection of the ophthalmic nerve (V1) and trigeminal ganglion (TG) 4 weeks after stereotactic electrocautery at (0 and 3 weeks) demonstrated a cavitating lesion of the distal V1 prior to entering the orbit (A; injury site). Distal to the injury, the nerve appeared darkened and grey in comparison to the contralateral V1, which retained its pale yellow appearance. Harvest of V1 and H&E staining demonstrated hypercellularity of the injury site (B) in comparison to the contralateral uninjured V1 (C) with loss of the microfasicular structure of the distal nerve on the side of injury (D) in comparison to the normal appearance of the V1 branches (E). This corresponded to a loss of GFP+ signal distal to the injury site (F) while GFP+ axons are clearly visible distal to the TG in the uninjured V1 (G). (Scale bar = 2000 µm in B/C; Scale bar = 500 µm in D-G).
3.5 Discussion

The findings in this study demonstrate that stereotactic electrocautery of the ophthalmic nerve (V1) effectively ablates the corneal innervation. However, some nerve regeneration and corneal reinnervation did occur four weeks after injury with considerable variability between rats. In order to improve the reliability of stereotactic electrocautery of the ophthalmic nerve and ensure that minimal corneal reinnervation occurred from the native corneal innervation four weeks after stereotactic electrocautery, we performed stereotactic electrocautery in the same location three weeks after the initial procedure. Complete corneal denervation is important for the future development of a rat model of corneal neurotization in order to be confident that any differences found are not due to the minimal reinnervation of the cornea by the native corneal innervation. Two episodes of stereotactic electrocautery of V1 effectively denervated the cornea, and all rats that underwent V1 stereotactic electrocautery developed corneal epithelial ulcerations and perforations. These changes are consistent with the development of neurotrophic keratopathy (NK) in these rats, suggesting that our rat model of neurotrophic keratopathy is sufficient as the basis for the development of an animal model of corneal neurotization.

Designing a model of NK with sustained corneal denervation was a challenge, as previous techniques had not been optimized to prevent corneal nerve regeneration after ablation. This is understandable, as previous studies had investigated only the effects of corneal denervation and were therefore only interested in acutely denervating the cornea and uninterested in any potential regeneration several weeks later (Ferrari et al. 2011; Namavari et al. 2011). The technique described by Yamaguchi et al. (2013) was attractive as a means of corneal denervation because it did not require specialized equipment, such as electrocautery or a stereotactic frame, and could be performed with standard surgical equipment. Unfortunately, we were unable to reliably identify the long ciliary nerves in the albino thy1-GFP+ SD rats. However, even with complete transection of all tissues adjacent to the optic nerve, corneal reinnervation was appreciable by two weeks with this technique. Corneal reinnervation was also observed past two weeks by Yamaguchi et al. although this was not reported in the paper (personal communication).

Corneal reinnervation was reduced with the use of topical ethanol (EtOH), which is known to be toxic to nerves and has been used in the past to damage the trigeminal nerve, causing NK (Davies 1970). However, EtOH also compromised the vasculature to the surface of the eye.
resulting in the development of cataracts in several rats. As a robust blood supply is necessary for the survival of nerve grafts after corneal neurotization, we abandoned this technique as model of NK for the purposes of investigating corneal neurotization.

We subsequently turned our attention to stereotactic techniques as they had been described extensively in the literature by several groups in rats (Nagano et al. 2003; Wong et al. 2004) and mice (Ferrari et al. 2011) with a high degree of reliability and success. Unlike Nagano and Wong who both used thermocoagulation, we used electrocautery, as was described by Ferrari et al (2011), because we had access to only an electrosurgical generator. Attempts to ablate the cell bodies of the corneal nerves, which are located in the TG, proved unsuccessful due to the unacceptable post-operative morbidity in the rats, including partial paralysis, poor oral intake, and weight loss. In contrast, stereotactic ablation of the V1, including performing the procedure twice, was well tolerated by all rats with very little post-operative morbidity (i.e. the rats continued to behave normally and gain weight after the procedure). We expect that these differences are due to the location of the V1 nerve which is further from the brain stem, resulting in less collateral damage to a vital structure. Unlike the TG, the V1 is also located deeper in the middle cranial fossa where a dense layer of meninges surrounds it superiorly and bone medially and laterally. This layer may insulate the zone of injury with less current spread to the cerebral spinal fluid (CSF) and brain.

The source of corneal reinnervation after stereotactic electrocautery was not investigated although we suspect this to be a combination of axons regenerating from the site of injury and perhaps sprouting from sympathetic and parasympathetic fibers in the corneal limbus. Considering that we found that a second procedure of V1 stereotactic electrocautery greatly reduced corneal innervation, it is likely that a portion of reinnervation derived from regenerating axons from the injured V1. We cannot rule out reinnervation by sympathetic and parasympathetic nerve fibers because we did not specifically address the sympathetic and parasympathetic innervation to the eye because their contribution is minor to the cornea. However, V1 ablation by stereotactic electrocautery did result in complete loss of GFP+ axons in the cornea. Furthermore, good evidence exists that parasympathetic and sympathetic innervation do not contribute to the trophic support of the corneal epithelium and loss of the sympathetic and parasympathetic innervation is known not to cause NK (Zander & Weddell 1951; Müller et al. 2003). Therefore, we felt there was no rationale to subject the rats to further surgery. Our method
is consistent with the surgeries performed in other animal models of NK, which ablate only the sensory innervation to the cornea derived from the TG (see Chapter 1.6).

Only rats in which successful stereotactic electrocautery of V1 was confirmed by an absent blink reflex to pressure (Cochet-Bonnet esthesiometry) and cold saline were used in these studies. Five percent of rats maintained a blink response after stereotactic electrocautery of V1, signifying that V1 remained intact and these rats were removed from analysis. Possible failure of the procedure could be due to malposition of the electrode as a result of aberrant anatomy or incorrect calculation of the stereotactic coordinates.

Except in rats that were observed for the development NK, demonstrated by corneal epithelial breakdown and corneal perforation, all rats received a complete surgical tarsorrhaphy (closure of the eyelids) in order to protect the cornea from injury. This was necessary, as leaving the cornea exposed resulted in advanced NK with rats developing corneal ulcerations, scarring and perforation within seven days of stereotactic electrocautery of V1. Maintaining the integrity of the cornea is necessary to examine corneal nerve regeneration with whole mount microscopy at one, two and four weeks after corneal denervation. This would have been impossible had the cornea been left unprotected as the cornea would have become extensively scarred, and rats in which the cornea had perforated had to be euthanized.

We have demonstrated that stereotactic electrocautery of V1 produces a reliable injury to the corneal innervation that results in complete corneal denervation one week after injury. Even with stereotactic electrocautery of V1, some corneal reinnervation is apparent four weeks after injury. Therefore, to ensure that no nerves from V1 reinnervate the cornea for the duration of four weeks, a second procedure of stereotactic electrocautery of V1 was performed three weeks later. This ensures that one week after the second procedure (i.e. four weeks after the initial procedure of stereotactic electrocautery) the cornea is not innervated by any axons from the original corneal innervation. The complete corneal denervation obtained with this rat model, as well as the induction of NK in rats treated with this method, make stereotactic electrocautery of V1 an appropriate model of NK in the rat to serve as the basis for a model of corneal neurotization.
Chapter 4  Development and Validation of a Rat Model of Corneal Reinnervation

This chapter is modified from the following:

4.1 Abstract

Purpose: Corneal neurotization is a novel surgical procedure that uses nerve grafts and functioning donor sensory nerves to reinnervate the cornea in patients with neurotrophic keratopathy (NK). Here we describe the development of a rat model of NK and corneal neurotization to investigate how corneal reinnervation from donor nerve fibers influences corneal epithelial maintenance and repair.

Methods: Thy1-GFP+ Sprague Dawley (SD) rats, which express green fluorescent protein in all axons, were used to develop the model. Corneal denervation was performed via stereotactic electrocautery of the ophthalmic nerve. Absent blink reflex was used to confirm corneal denervation. Corneal neurotization was performed using a sural and common peroneal (CP) nerve autograft and the contralateral infraorbital nerve as a donor to reinnervate the cornea. Corneal imaging including nerve density measurements, and retrograde labeling were performed to compare corneal innervation in rats with corneal neurotization (after ophthalmic nerve electrocautery) to rats with ophthalmic nerve electrocautery alone and the uninjured (normal) corneal innervation. Corneal epithelial healing was compared between the three groups using an in vivo corneal healing assay.

Results: Four weeks after corneal denervation, rats with corneal neurotization demonstrated significantly higher corneal nerve density (62872 µm/mm² ± 12400) in comparison to rats with only ophthalmic nerve electrocautery (2301 µm/mm² ± 1347; p < 0.01). Retrograde-labeling of the cornea in rats with corneal neurotization labeled 206 ± 82 neurons in the contralateral trigeminal ganglion, confirming axons reinnervating the cornea derived from the contralateral infraorbital nerve. In rats with corneal denervation, corneal reinnervation after corneal neurotization significantly improved healing after corneal injury in comparison to rats without corneal neurotization (p < 0.01).

Conclusions: Donor nerve fibers reinnervate the cornea after corneal neurotization and significantly improve corneal epithelial healing after injury. This model can be used to further investigate how corneal neurotization influences epithelial maintenance and repair in the context of NK.
4.2 Introduction

Corneal sensation protects the eye from injury and the corneal nerves produce trophic mediators necessary to maintain and repair the corneal epithelium (Müller et al. 2003; Shaheen et al. 2014). Absent corneal innervation is congenital or arises secondary to traumatic, iatrogenic, metabolic or infectious injury to the ophthalmic branch of the trigeminal nerve, which innervates the cornea. Patients with impaired corneal innervation develop neurotrophic keratopathy (NK), which is characterized by breakdown, ulceration and poor healing of the corneal epithelium (Sigelman & Friedenwald 1954; Alper 1975; Cavanagh & Colley 1989; Beuerman & Schimmelpfennig 1980; Baker et al. 1993; Chan & Haschke 1982; Chan & Haschke 1981; Ferrari et al. 2011; Ueno et al. 2012). Recurrent and persistent corneal epithelial breakdown in patients with NK inevitably progresses to corneal scarring and opacification, resulting in permanent, irreversible vision loss (Rosenberg 1984; Ramaesh et al. 2007; Lambley et al. 2014; Sacchetti & Lambiase 2014; Agranat et al. 2016). NK remains one of the more difficult ophthalmic conditions to treat and a leading cause of corneal blindness worldwide (Sacchetti & Lambiase 2014). Conventional ophthalmic management often fails to prevent vision loss in patients with NK as established ophthalmic treatments do not to address the underlying absence of corneal innervation and loss of nerve-derived trophic support.

Mediators derived from the corneal innervation are essential for the maintenance and healing of the corneal epithelium (Müller et al. 2003; Shaheen et al. 2014). Immediately after loss of the corneal innervation, there is thinning, breakdown and ulceration of the corneal epithelium (Sigelman & Friedenwald 1954; Alper 1975; Cavanagh & Colley 1989; Beuerman & Schimmelpfennig 1980). The corneal denervation that arises from loss of the corneal innervation also significantly impairs healing of the cornea epithelium after injury, (Beuerman & Schimmelpfennig 1980; Schimmelpfennig & Beuerman 1982; Araki et al. 1994; Gallar et al. 1990; Ferrari et al. 2011) possibly in part because of decreased limbal stem cell proliferation and migration (Ueno et al. 2012). Neuromediators found in the corneal epithelium, such as nerve-derived growth factor and Substance P, have been used topically to improve healing of persistent corneal epithelial ulcerations in patients with NK (Lambiase et al. 1998; Yamada et al. 2008). However, patients are left dependent on lifelong drug treatment and never recover protective
sensation necessary for mechanical protection (Soni & Jeng 2016; Yamada et al. 2008; Lambiase et al. 1998).

Surgical reinnervation of the cornea with donor nerves (i.e. corneal neurotization) restores innervation and sensation to the cornea in patients with NK, thereby addressing the underlying pathophysiology in NK (Samii 1981; Terzis et al. 2009; Elbaz et al. 2014). Reinnervation of the cornea by donor nerves after corneal neurotization in patients with NK has been demonstrated definitively with histology, magnetoencephalography and \textit{in-vivo} confocal microscopy (see Chapter 3). In a prospective study, corneal neurotization improved corneal sensation, decreased the incidence of persistent corneal epithelial breakdown and prevented further vision loss in patients with NK (see Chapter 3). This suggests that, in addition to improving sensation, donor nerves that reinnervate the cornea after corneal neurotization contain neuromediators that are essential to prevent breakdown of the corneal epithelium. However, patients in these studies received conventional ophthalmic treatment in addition to corneal neurotization and therefore due to these limitations it remains unknown whether corneal neurotization alone improves corneal epithelial maintenance and repair.

Randomized controlled trials in patients with NK are difficult because of the rarity and heterogeneity of the disease. Yet further investigation is necessary to determine whether donor nerves that reinnervate the cornea after corneal neurotization contain the essential neuromediators supplied by the native corneal innervation. The objective of this study was to develop the first animal model of NK and corneal neurotization. An animal model is necessary to investigate corneal neurotization as a surgical technique to prevent vision loss and blindness in patients with NK.
4.3 Materials and Methods

4.3.1 Study Design

A rat model of neurotrophic keratopathy (NK) and corneal neurotization (CN) was developed in the rat using a Thy1-GFP+ Sprague Dawley (SD). The Thy1-GFP+ SD rat strain was used to develop the model because they express green fluorescent protein in all axons, permitting visualization of axons in the cornea without the need of immunohistochemistry (Figure 3-1).

After developing a method of corneal neurotization in the rat a preliminary experiment with 8 rats was conducted to determine appropriate time-points for CN relative to corneal denervation (i.e. the length of time required for donor axons to grow into the cornea after neurotization). The remaining experiments, describing the corneal nerve density, retrograde labeling, histomorphometry and investigating healing were conducted with the same time-points for CN determined in our preliminary experiments described below.

4.3.2 Animals

Fifty female Thy1-GFP+ Sprague Dawley rats (250 – 300 g) were used. Thy1-GFP+ rats express green fluorescent protein in all axons permitting the visualization of the native corneal innervation and reinnervation of the cornea with corneal neurotization after corneal denervation. All rats were maintained in a temperature and humidity controlled environment with a 12:12 h light:dark cycle and received ad lib water and standard rat chow (Purina, Mississauga, ON). Surgical procedures were conducted in an aseptic manner with an operating microscope (Leitz, Willowdale, ON). Rats were sacrificed at study termination under deep anesthesia using intraperitoneal (i.p) Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg, Bimeda-MTC, Cambridge, ON). The experiments were approved by The Hospital for Sick Children Laboratory Animal Services, which adheres to the guidelines of the Canadian Council on Animal Care.
Eight rats were used to determine the appropriate time-point (4.3.3) for ophthalmic nerve ablation and corneal reinnervation with corneal neurotization. For model validation, four rats in each group were included for corneal axon density (4.3.6) and graft histomorphometry (4.3.8). A separate group of four rats had to be used for retrograde labeling (4.3.7) because the corneal epithelial debridement required for labeling disrupts the subbasal axon morphology. Six rats in each group were used to investigate corneal healing (4.3.9)

4.3.3 Surgical Procedures: Stereotactic Electrocautery of V1 and Corneal Neurotization

All surgical procedures were conducted in an aseptic manner with an operating microscope (Leitz, Willowdale, ON) under inhalational anesthetic (2% isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ).

*Stereotactic Electrocautery of the Ophthalmic Nerve (i.e. Corneal Denervation):* Ablation of the native corneal innervation (i.e. corneal denervation) was performed using stereotactic electrocautery of the ophthalmic nerve, which contains the corneal innervation, as previously described in mice (Ferrari et al. 2011) and rats (Nagano et al. 2003). Rats were mounted on a stereotactic frame (Harvard Apparatus, Hollingston, Massachusetts) and a midline cranial incision was made to identify the bregma (intersection point of the coronal and sagittal sutures). A 1 mm burr hole was made at the coordinates AP + 1.5 mm; ML + 2.0 mm. Exact coordinates were confirmed with dissection on rat cadavers. An insulated 22 G monopolar electrode (UP 3/50, Pajunk GmbH, Germany), with 1 mm of insulation removed from the tip, was lowered to a depth of 10 mm through the burr hole. An electrosurgical generator (Force FCTM-8C, Medtronic, USA) was then used to ablate the ophthalmic nerve (10 W for 60 s). The electrode was then removed and the skin sutured. A complete tarsorrhaphy (suturing together the eyelids) was performed to protect the denervated cornea and rats were provided with burprenorphine (1 mg/kg; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) for post-operative pain relief. Ablation of the corneal innervation and absent sensation were confirmed by an absent blink reflex to touch and cold saline under light anaesthesia in comparison to the contralateral eye. Stereotactic electrocautery of the ophthalmic nerve, as described above, was repeated three
weeks later to ensure that no regeneration of the native corneal innervation had occurred four weeks after the initial stereotactic procedure, which is when tissue analysis was performed.

**Corneal Neurotization:** Corneal neurotization in the thy1-GFP+ rat was performed using the contralateral infraorbital nerve as a donor and two nerve grafts harvested from the left common peroneal and sural nerves (Figure 4-1).

Briefly, two nerve grafts, a common peroneal (CP) nerve graft and sural nerve graft, were harvested from the left leg of the rat. A curvilinear incision was made over the left femur and left tibia extending from the mid-femur to the distal ankle. The biceps femoris was dissected from the quadratus femoris muscle to expose the sciatic nerve. This was followed distally to expose the entire length of the CP nerve. The biceps femoris muscle was then dissected off the knee and separated from the anterior compartment of the lower leg and the gastrocnemius muscle in order to visualize the entire length of the sural nerve (Figure 4-2 A). The entire length of the CP and sural nerves were harvested in order to provide sufficient length for the grafts to span the distance from the right (contralateral) ION to the left cornea. The sural and CP nerve grafts measured approximately 30 mm after harvest (Figure 4-2 B). The biceps femoris fascia was then repaired using 4-0 vicryl suture and the skin incision closed using 5-0 vicryl suture.

Attention was then turned to exposure of the right (contralateral) ION. A 5 mm linear incision was made over the proximal edge of the whisker pad. Dissection through the superficial musculature exposed the origin of the ION from the infraorbital foramen. The ION was dissected free from surrounding tissue at the infraorbital foramen, and was then dissected distally as it traveled underneath the whisker pad. Approximately 2 to 3 mm from the infraorbital foramen, branches of the facial nerve could be seen coursing over the ION. These were transected proximally and excised. The ION dissection was carried out as distally as possible, at which point the ION was transected distally and freed from the surrounding tissue in preparation for coaptation to the sural and CP nerve grafts (Figure 4-3 A).

A subcutaneous tunnel was then dissected from the right (contralateral) ION towards the inferior and superior conjunctiva of the left eye. A small 2 mm incision was then made in the superior and inferior conjunctiva of the left eye and this was bluntly dissected to connect with the subcutaneous tunnel spanning from the right (contralateral) ION. The sural and CP nerves were then placed in the incision by the contralateral ION and the proximal end of the nerve grafts were
pulled through the tunnel into the inferior and superior conjunctival incisions respectively over the left eye (Figure 4-3 B). The distal end of the each nerve graft was separately coapted to the right (contralateral) ION (Figure 4-3 C).

A second curvilinear incision was made into the inferior and superior perilimbal conjunctiva of the left eye and the sural and CP nerve grafts were tunnel below the conjunctival flaps into the perilimbal space (Figure 4-4 A). The ends of the CP and sural nerve grafts were then both trimmed and sutured to the superior and inferior corneal-scleral junction respectively with 9-0 nylon suture (Figure 4-4 B). The overlying conjunctival incisions were then closed to protect the nerve grafts from desiccation and promote revascularization of the grafts (Figure 4-4 C).

Post-operatively, the ocular surface was protected with a tarsorrhaphy, using a 6-0 polypropylene suture, to allow the ocular surface to heal and protect the cornea from injury. The contralateral facial incision was closed with a 5-0 Vicryl suture.

**Figure 4–1 Corneal Neurotization in the Rat.** Corneal neurotization was performed of the left rat cornea using the contralateral infraorbital nerve (ION) as a donor source of axons to reinnervate the
cornea. Regenerating axons from the transected ION were guided into the left cornea via two nerve grafts, which were independently coapted to the contralateral ION. The nerve grafts were then tunneled subcutaneously and below the conjunctiva via superior and inferior conjunctival incisions. The common peroneal nerve graft was then tunneled below the conjunctiva into a perilimbal incision in the superior orbit and sutured directly to the superior limbus. In similar fashion, the sural nerve was then tunneled below the conjunctiva into a perilimbal incision in the inferior orbit and sutured directly to the inferior limbus. This provides a pathway for axons to regenerate from the transected ION to the left cornea.

Figure 4–2 Harvest of the Sural and Common Peroneal Nerve Grafts. Two nerve grafts were harvested from the common peroneal (CP) and sural nerves. The entire length of the CP and sural nerves were exposed by dissecting the biceps femoris away from the quadratus femoris, tibialis anterior and gastrocnemius muscles. Approximately 30 mm of CP and 28 mm of sural nerve were available for harvest. Both grafts were of sufficient length to span the distance from the contralateral infraorbital nerve coaptation to the left cornea.
Figure 4–3 Dissection of the Infraorbital Nerve and Coaptation. The proximal right infraorbital (ION) nerve was identified through a linear incision proximal to the whisker pad at the infraorbital foramen. The nerve was then dissected distally into the whisker pad where it was transected at it’s most distal innervation and rotated medially towards the contralateral eye (A; the proximal and distal ION are demonstrated by the green arrows). A subcutaneous tunnel was then made from the ION towards the contralateral superior and inferior conjunctiva. Two curvilinear incisions were made into the superior and inferior conjunctiva and these were connected to the subcutaneous tunnel. The proximal end of the common peroneal (CP) and sural nerve grafts were then tunneled from the ION into the conjunctival incision and brought over the left eye (B; yellow arrow demonstrates the CP nerve graft; blue arrow demonstrates the sural nerve graft). The distal nerve ends of the nerve grafts were then coapted to the transected ION (C).

Figure 4–4 Coaptation of the Nerve Grafts to the Corneal-Scleral Junction. The sural and CP nerve grafts were tunneled below the conjunctiva into a conjunctival incision 1 mm adjacent to the corneal-scleral junction (A). The conjunctiva was then dissected towards the cornea to expose the corneal-scleral junction. The nerve graft was the trimmed and sutured directly to the sclera adjacent to the limbus with 9-0 nylon suture (B). The conjunctival incisions were then closed over the nerve graft to prevent desiccation and promote revascularization. The CP nerve graft is highlighted by the yellow arrows.
4.3.4 Determination of Appropriate Timing for Corneal Neurotization and Stereotactic Ablation of the Corneal Innervation

Due to the length of the nerve grafts, measuring approximately 30 mm, corneal neurotization was performed prior to stereotactic electrocautery of the ophthalmic nerve to provide regenerating axons from the ION with time to regenerate through the nerve grafts to reach the left cornea. The length of time for axons to reinnervate the cornea from the site of coaptation at the right (contralateral) ION was determined. Eight rats received corneal neurotization at Day 0, and were then randomized to receive ophthalmic nerve electrocautery (i.e. corneal denervation) at either 4 or 6 weeks after corneal neurotization, permitting time for the regenerating grafts to grow through the 30 mm nerve grafts (Table 4-1). Two animals in each group were then harvested 2 or 4 weeks after ophthalmic nerve electrocautery to investigate the time required for axons to reinnervate the cornea after corneal denervation. The entire cornea was imaged to evaluate the amount of corneal reinnervation.

Table 4–1 Determination of Time-point Required for Corneal Reinnervation. Corneal neurotization was performed prior to ophthalmic nerve electrocautery to allow regenerating axons to regenerate across the 30 mm length of nerve graft. Performing corneal neurotization prior to ophthalmic nerve electrocautery also allows the ocular surface to heal prior to the induction of neurotrophic keratopathy. Based on a rate of axon regeneration of approximately 1 mm/day and a delay of up to 2 weeks for axons to cross the coaptation site, we hypothesized that 4 to 6 weeks was an appropriate time point for axons to reach the corneal-scleral junction. Rats were randomized to receive ophthalmic nerve electrocautery at either 4 or 6 weeks after corneal neurotization. Once the corneal nerves were ablated, the length of time for corneal reinnervation from the donor infraorbital nerve source was also unknown. Therefore, half of the animals in each group were harvested either 2 or 4 weeks after ophthalmic nerve electrocautery to determine the density of corneal reinnervation. This preliminary experiment allowed us to determine the appropriate time-points for corneal neurotization and ophthalmic nerve electrocautery in order to achieve robust reinnervation of the cornea.
4.3.5 Ophthalmic Nerve Gross Pathology and Histology

Injury to the ophthalmic nerve after V1 electrocautery was confirmed with cadaveric dissections and histology. Rats were perfused with normal saline and 4% paraformaldehyde (PFA). The trigeminal ganglion and ophthalmic nerve were exposed and digital photographs were taken of the lesion site. After removing the surrounding tissues, the trigeminal ganglion and ophthalmic nerve were harvested and stained with hematoxylin and eosin and examined confocal microscopy.

4.3.6 Quantification of Corneal Nerve Density

Four weeks after stereotactic electrocautery of the ophthalmic nerve, whole globes were harvested and immersed immediately in 0.2 % picric acid and 4 % PFA dissolved in 0.1 M PBS for 30 minutes. Corneas were dissected from the globe with a scalpel and returned to the fixative solution for 90 minutes, washed and stored in 30 % sucrose in 0.1 M PBS for 24 to 48 hours until clear. Thereafter, corneas were cut into four corneal quadrants and mounted onto Superfrost slides (Fisher Scientific, Ottawa, ON).

The whole mount corneal slides were visualized using a confocal microscope (Olympus IX81) with a 10 x objective. A minimum of three locations distributed evenly in the peripheral cornea and two images from the central cornea were imaged with 1 µm Z-stacks of the entire corneal thickness. All the images taken were used for analysis. Z-stacks were analyzed using Volocity software (Perkin-Elmer, Waltham, MA) to separate images into the stromal, subbasal and epithelial layers of the cornea. Images were analyzed separately using ImageJ and NeuronJ
plugin to calculate corneal nerve density (in $\mu$m/mm$^2$) as described previously (Yamaguchi, Turhan, Deshea L. Harris, et al. 2013). Briefly, images were imported into NeuronJ and the entire length of each GFP+ axon in the image was traced to calculate the total nerve length and the axon density for each image. Additionally, the entire whole mount corneal slides were imaged using a confocal microscope (Olympus IX81) and 100 $\mu$m z-stacks (with 10 $\mu$m slice thickness) to visualize and determine the extent of corneal reinnervation of the entire cornea. Images were stitched using Volocity software (Perkin-Elmer, Waltham, MA) and image scales were set to produce an entire image of the corneal innervation for analysis of corneal reinnervation after corneal neurotization.

### 4.3.7 Corneal Retrograde Labeling

Retrograde-labeling of the cornea was performed four weeks after initial stereotactic ablation of the ophthalmic nerve. The following protocol was modified from previous reports of retrograde-labeling (Ivanusic et al. 2013; López de Armentia et al. 2000; De Felipe et al. 1999). Filter paper (4 mm in diameter) was soaked in 70% ethanol and positioned on the center of the corneal surface and left in place for 30 seconds. The disc was then withdrawn and the corneal epithelium removed with a No. 15 scalpel blade. Immediately afterwards, a piece of absorbable gelatin sponge (Gelfoam, Pfizer Canada Inc., Kirkland, Canada) soaked in 4% FluoroGold (FG: Fluorochrome LLC, Denver, CO) was placed on the wounded area for 1 hour. The cornea and wound were rinsed three times with sterile saline and the rats returned to their cages.

Rats were sacrificed seven days after retrograde-labeling and were euthanized using intraperitoneal Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg, Bimeda-MTC, Cambridge, ON) and perfused with 4% paraformaldehyde (PFA). The TGs of both the injured left (ipsilateral) and uninjured right (contralateral) ophthalmic nerve were harvested and post-fixed in 4% PFA for one day and then cryoprotected in 30% sucrose in 4% PFA for four days prior to embedding in optimal cutting temperature compound (OCT: Sakura Fine Technical Co., Torrence, CA). TGs were serially sectioned at 20 $\mu$m using a cryostat (Leica Microsystems Inc., Concord, ON) at -22$^\circ$C and mounted onto Superfrost slides (Fisher Scientific, Ottawa, ON). Retrograde-labeled sensory neurons in trigeminal ganglia were counted on an epifluorescent microscope with a 10x objective (100x overall magnification; Leica). A blinded observer
performed all counts and a correction for double counting was made using a correction factor previously described by Abercrombie (1946) (Abercrombie 1946).

4.3.8 Nerve Graft Histomorphometry

Nerve samples, 3 mm in length, of both the sural and CP nerve grafts were harvested prior to sacrifice for nerve histomorphometry. Nerve samples were fixed in 2.5% glutaraldehyde and buffered in 0.025 M cacodylate overnight, washed, and then stored in 0.15 M cacodylate buffer. The samples were fixed in 2% osmium tetraoxide, washed in graded alcohols, and embedded in EPON. Transverse sections at 1 µm thickness, were made through the center of the nerve sample and stained with toluidine blue. The observer was blinded to the identity of the experimental group. The nerve cross-sections were photographed under light microscopy (1000x) using Image Pro Plus software (MediaCybernetics, Bethesda, MD, USA) and the images analyzed using MATLAB software (Mathworks Inc., Natick, Mass., USA).

4.3.9 Assessment of Corneal Healing after Injury to the Corneal Epithelium

Four weeks after the initial stereotactic electrocautery of the ophthalmic nerve, rats with and without corneal neurotization were anesthetized using inhalational anesthetic (2% Isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ). The cornea was first assessed for a blink reflex with cold saline and a corneal esthesiometer (Luneau Ophthalmologie, Chartres, France). With a surgical microscope, the corneal epithelium was carefully removed with a 0.5 mm burr using the Algerbrush II (Alger Company Inc., Lago Vista, Texas). Fluorescein staining (DioFluor™ Strips, Innova Medical Ophthalmics Inc., Toronto, Canada) and digital imaging (Nikon D 5100) was performed immediately and thereafter every 12 hours up to 96 hours after injury to monitor wound size and healing of the corneal epithelium. Imaging was performed with a standardized frame keeping the camera a fixed distance from the ocular surface. Wound size was calculated using ImageJ and healing standardized to the initial wound size. Comparison of corneal healing was made to rats with intact, normal corneal sensation.
4.3.10 Statistical Analysis

All statistical analysis was performed using GraphPad Prism® version 6.0 for Mac (GraphPad Software, Inc; San Diego, California). All data were analyzed using a one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. Wound size was analyzed using one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction at 96 hours as well as a repeated measures ANOVA over time. Statistical significance was accepted at the level of $p < 0.05$; all data are expressed as the mean ± standard deviation (SD).
4.4 Results

4.4.1 Corneal Reinnervation after Corneal Neurotization

Corneal harvest two weeks after corneal neurotization and stereotactic electrocautery of the ophthalmic nerve, demonstrated minimal corneal reinnervation, suggesting that two weeks was insufficient for donor axons to reinnervate the cornea after ophthalmic nerve electrocautery (Figure 4-5 A/B). Significantly more GFP+ axons were appreciable in the cornea when corneal harvest was performed four weeks after ophthalmic nerve electrocautery (Figure 4-5 C/D), demonstrating that corneal harvest four weeks after corneal denervation is required to permit donor nerves regenerating from the donor infraorbital nerve to reinnervate the cornea. Performing ophthalmic nerve electrocautery six weeks after corneal neurotization resulted in greater corneal reinnervation than when ophthalmic nerve electrocautery was performed four weeks after corneal neurotization. Ophthalmic nerve electrocautery six weeks after corneal neurotization and corneal harvest four weeks after ophthalmic nerve electrocautery resulted in the greatest amount of corneal reinnervation, therefore these time-points were used for all other experiments in this chapter.

It is important to note that donor axon regeneration into the cornea was only possible after corneal denervation when the native corneal innervation had been ablated with ophthalmic nerve electrocautery. When ophthalmic nerve electrocautery was performed 9 weeks after corneal neurotization and the cornea harvested one week afterwards (i.e. 10 weeks after corneal neurotization), there was minimal corneal reinnervation (Figure 4-6). This result demonstrates that corneal denervation is necessary for donor axons to reinnervate the cornea.
Figure 4–5 Corneal Reinnervation after Corneal Neurotization. Minimal corneal reinnervation was found when corneas were harvested 2 weeks after ophthalmic nerve electrocautery regardless of whether corneal neurotization had occurred four (A) or six (B) weeks prior to ophthalmic nerve electrocautery. Corneal reinnervation was significantly higher when axons were provided with 4 weeks to regenerate into the cornea after ophthalmic nerve electrocautery when corneal neurotization was performed both four (C) and six (D) weeks prior to ophthalmic nerve electrocautery. Corneal reinnervation was consistently greater in rats receiving electrocautery six weeks after corneal neurotization, therefore the following time-point was used for all future experiments: corneal neurotization followed by ophthalmic nerve electrocautery 6 weeks later, with corneal harvest and tissue analyses 4 weeks after ophthalmic nerve electrocautery.
When ophthalmic nerve electrocautery was performed 9 weeks after corneal neurotization and the cornea was examined 1 week afterwards (a total of 10 weeks after corneal neurotization), minimal corneal reinnervation had occurred (A/B). Significantly more corneal reinnervation was visible 10 weeks after corneal neurotization after the corneal innervation had been ablated for 4 weeks (Fig 5-5 C/D), suggesting the absence of corneal nerves after ophthalmic nerve electrocautery is necessary for axons derived from the nerve grafts to grow into and reinnervate the cornea.

**4.4.2 Corneal Neurotization Increases Corneal Axon Density in Rats with Neurotrophic Keratopathy**

Four weeks after stereotactic electrocautery of the left ophthalmic nerve, the left cornea demonstrated near complete loss of nerve fibers in the cornea (Figure 4-7A-D). There was obvious reinnervation of the cornea four weeks after stereotactic electrocautery of the ophthalmic nerve in rats that had received corneal neurotization (Figure 4-7E-H).

Ophthalmic nerve electrocautery resulted in almost complete loss of sub-basal (101 µm/mm² ± 203) and stromal (874 µm/mm² ± 990) axons in the center of the cornea in comparison to the
sub-basal (26,486 µm/mm² ± 3269; \( p < 0.0001 \)) and stromal (20,480 µm/mm² ± 5569; \( p < 0.0001 \)) central corneal innervation in uninjured rats. The peripheral sub-basal (285 µm/mm² ± 142) and stromal (1,270 µm/mm² ± 771) innervation was also significantly reduced in comparison to the peripheral sub-basal (22,458 µm/mm² ± 4421; \( p < 0.0001 \)) and stromal (19,470 µm/mm² ± 2254; \( p < 0.0001 \)) innervation in uninjured rats. In rats with V1 electrocautery, this represents a 99.6 % and 95.7 % reduction of the central sub-basal and stromal corneal innervation and a 98.7 % and 93.5 % reduction of the peripheral sub-basal and stromal corneal innervation.

In rats with corneal neurotization prior to ophthalmic nerve electrocautery, central sub-basal (48,567 µm/mm² ± 11987; \( p = 0.0068 \)) and stromal (20,223 µm/mm² ± 2877; \( p < 0.0001 \)) corneal innervation were significantly increased in comparison to rats receiving only V1 electrocautery. CN also significantly increased peripheral sub-basal (26,405 µm/mm² ± 1692; \( p < 0.0001 \)) and stromal (26,085 µm/mm² ± 1464; \( p < 0.0001 \)) corneal innervation. In comparison to the uninjured (normal) corneal innervation, corneal innervation in rats with CN prior to V1 electrocautery was not statistically different. These results are summarized in Figure 4-8 and 4-9.
Figure 4–7 Corneal Reinnervation After Corneal Neurotization in Comparison to Ophthalmic Nerve Electrocautery. Ophthalmic nerve electrocautery at 4 weeks and then again at 1 week prior to corneal harvest resulted in almost complete loss of GFP+ corneal axons in the peripheral cornea, and only one rat demonstrated very few axons in the central cornea (A-D). In contrast, rats receiving corneal neurotization demonstrated significantly greater GFP+ corneal axons in the central and peripheral cornea (E-H).
A

Uninjured

Neurotized

Denervated

B

Central Cornea Axon Density

Axon Density (μm/mm²)

Uninjured Control
Neurotized
Denervated

* p < 0.01
**Figure 4–8 Central Corneal Nerve Density.** Imaging of the central cornea demonstrated near complete loss of GFP+ axons in the stroma and complete loss of central sub-basal axons after ophthalmic nerve electrocautery (i.e. “denervated”) (A). Corneal neurotization (i.e. neurotized) rats demonstrated significantly increased density of GFP+ axons in the sub-basal and stromal cornea, and this was comparable to the uninjured normal corneal innervation (i.e. “uninjured”) (A). Quantification of axon density as the total nerve fiber length (µm) per area (mm$^2$) demonstrated that the sub-basal and stromal corneal innervation in rats with corneal neurotization prior to ophthalmic nerve electrocautery was significantly higher than rats with only ophthalmic nerve electrocautery and comparable to the uninjured normal cornea. (Scale bar = 44 µm)
A

Uninjured

Neurotized

Denervated

B

Peripheral Cornea Axon Density

Axon Density (μm/mm²)

Subbasal  |  Stromal

Uninjured Control  |  Neurotized  |  Denervated

* p < 0.01
**Figure 4–9 Peripheral Corneal Nerve Density.** Imaging of the peripheral cornea demonstrated near complete loss of GFP+ subbasal and stromal axons after ophthalmic nerve electrocautery (i.e. “denervated”) (A). Corneal neurotization (i.e. “neurotized”) rats demonstrated significantly increased density of GFP+ axons in the peripheral sub-basal and stromal cornea, and this was comparable to the uninjured normal corneal innervation (i.e. “uninjured”) (A). Quantification of axon density as the total nerve fiber length (µm) per area (mm²) in images sampled from peripheral cornea, demonstrated that the sub-basal and stromal corneal innervation in rats with corneal neurotization was significantly higher than denervated animals and comparable to the uninjured normal cornea. (Scale bar = 44 µm)

4.4.3 Corneal Neurotization Reinnervates the Cornea with Axons Derived from the Contralateral Trigeminal Ganglion

Retrograde labeling data is summarized in Table 4-2. Retrograde-labeling of the normal (uninjured) left corneal innervation in *thy1*-GFP+ rats labeled neurons exclusively in the left (ipsilateral) trigeminal ganglion (TG) with no neurons in the right (contralateral) TG. Retrograde labeling of the left cornea four weeks after stereotactic electrocautery of the ophthalmic nerve demonstrated a significant decrease in the number of neurons innervating the cornea (*p* < 0.0001). Again all labeled neurons were found in the left (ipsilateral) TG and no neurons in the right (contralateral) TG. These findings are consistent with significant corneal denervation after stereotactic electrocautery of ophthalmic nerve.

In contrast, retrograde labeling of the left cornea four weeks after stereotactic ablation of ophthalmic nerve in rats with left corneal neurotization labeled almost no neurons in the left (ipsilateral) TG however a significant number of neurons in the right (contralateral) TG (Table 4-2). This finding demonstrates that nerve fibers reinnervating the cornea after corneal neurotization are derived from the right (contralateral) TG, which is consistent with the right (contralateral) infraorbital nerve that was used as a donor. There was no statistically significant difference in the total number of neurons innervating the left cornea (i.e. labeled neurons) in uninjured and neurotized rat corneas, while there was a significantly higher mean (+ SD) number of total neurons in both groups as compared to the number when the ophthalmic nerve was ablated with stereotactic electrocautery (*F*(2,9) = 21.78; *p* < 0.001). Representative images of the left and right TGs in a rat with CN is shown in Figure 4-10.
Table 4–2 Number and Location of Retrograde Labeled Neurons. RG-labeling of the left cornea with 4% FG was performed to determine the number and origin of axons innervating the cornea. Rats with ophthalmic nerve electrocautery were compared to uninjured controls (i.e. the normal corneal innervation) and rats that received corneal neurotization prior to ophthalmic nerve electrocautery. The ipsilateral (left) and contralateral (right) TG were harvested four weeks after ophthalmic nerve electrocautery, fixed and cut into 20 µm sections. Rats with corneal neurotization and rats with uninjured corneal innervation demonstrated a significantly greater number of neurons innervating the left cornea in comparison to rats with ophthalmic nerve electrocautery only ($p < 0.001$). However, in contrast to the normal corneal innervation, which derives completely from the ipsilateral (left) TG, the corneal innervation rats with corneal neurotization derived from the contralateral (right) TG confirming corneal reinnervation derived from the donor infraorbital nerve.

<table>
<thead>
<tr>
<th></th>
<th>Uninjured</th>
<th>V1 electrocautery</th>
<th>CN</th>
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</thead>
<tbody>
<tr>
<td>Left TG</td>
<td>219 ± 36</td>
<td>5 ± 3.9</td>
<td>1 ± 1.0</td>
</tr>
<tr>
<td>Right TG</td>
<td>0</td>
<td>0</td>
<td>206 ± 82.0</td>
</tr>
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</table>
Figure 4–10 Retrograde Labeling After Corneal Neurotization. RG-labeling with Fluorogold of the left cornea in rats with corneal neurotization resulted in almost no identifiably labeled neurons in the left TG (A), however labeled neurons (red arrows) were identifiable in the right (contralateral) TG (B). Labeled neurons can be seen under higher magnification (red arrows) in Panel D. Ophthalmic nerve electrocautery injury to the left ophthalmic nerve (yellow arrow in A and C) resulted in a cavitation injury and loss of the fascicular nerve architecture, in contrast to the uninjured (right) TG (D), where GFP+ axons are visible distal to the site of injury. This demonstrates that axons reinnervating the cornea after CN derive from the contralateral TG. (Scale bar = 500 µm)

4.4.4 Significantly More Axons Regenerate Through the CP and Sural Nerve Grafts than Reinnervate the Cornea

Histomorphometric analysis identified a significantly higher number of axons regenerating through the common peroneal (CP) nerve (5577 ± 647) in comparison to the sural nerve (2430 ± 613; p < 0.001). This represents a significantly higher number of axons regenerating through the grafts than were found to reinnervate the cornea with retrograde labeling (207 ± 81.5). Axons regenerating through the CP nerve tended to be smaller in axon diameter than axons regenerating through the sural nerve (1.37 ± 0.04 vs 1.81 ± 0.14; p < 0.0001), however there was no difference between the two in total fiber diameter (2.55 ± 0.10 vs 2.39 ± 0.25; p = 0.2796). This is consistent with greater myelination of axons regenerating through the CP nerve graft as axons in the CP demonstrated a higher G-ratio than axons regenerating through the sural nerve graft (0.5373 vs 0.4932). This data is summarized in Table 4-3.
Table 4–3 Nerve Graft Histomorphometry. Ten weeks after corneal neurotization of the left cornea, samples of the distal common peroneal and sural nerve grafts were harvested adjacent to the site of corneal neurotization in order to count the number of myelinated axons that had regenerated through the nerve grafts. A significantly higher number of myelinated axons were found to have regenerated through the nerve grafts (~ 9000) in comparison to the number of neurons (~ 200) found to reinnervate the cornea with retrograde labeling. (Mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Common Peroneal</th>
<th>Sural</th>
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</thead>
<tbody>
<tr>
<td>Axon Number</td>
<td>5577 ± 647</td>
<td>2430 ± 613</td>
</tr>
<tr>
<td>Axon Diameter</td>
<td>1.37 ± 0.04</td>
<td>1.81 ± 0.14</td>
</tr>
<tr>
<td>Fibre Diameter</td>
<td>2.55 ± 0.10</td>
<td>2.39 ± 0.25</td>
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<tr>
<td>Axon Area</td>
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</tr>
<tr>
<td>Total Fibre Area</td>
<td>5.5339</td>
<td>4.8771</td>
</tr>
<tr>
<td>G-ratio</td>
<td>0.5373</td>
<td>0.4932</td>
</tr>
</tbody>
</table>

4.4.5 Corneal Neurotization Improves Healing after Corneal Injury

Following a complete corneal de-epithelization wound, the corneal wound healed more quickly in rats with corneal neurotization after stereotactic ablation of the ophthalmic nerve than in rats with stereotactic ablation of the ophthalmic nerve alone. Representative images of the corneal wound in each group are shown in Figure 4-11A. In rats with normal (i.e. uninjured) corneal innervation, the corneal wound healed in all rats (n = 6) within 96 hours (mean 72 hours ± 9.2) (Figure 4-11B). After stereotactic ablation of the ophthalmic nerve, the corneas without corneal neurotization demonstrated impaired wound healing, with no cornea demonstrating complete closure of the corneal wound by 96 hours and all rats (n = 6). All rats with corneal denervation from stereotactic ablation of the ophthalmic nerve demonstrated corneal perforations prior to the 96 hour time point, which is a severe complication of NK.

The corneal wound in rats with corneal neurotization healed significantly more quickly than rats with stereotactic ablation alone, with a greater percentage of the wound demonstrating re-epithelization by 96 hours (88 % ± 9.7 vs 47 % ± 14.8; p < 0.01) (Figure 4-11B). Moreover, no rat with corneal neurotization developed a corneal perforation, and two rats (33 %) demonstrated complete wound healing by 96 hours.
Figure 4–11 Corneal Healing After Corneal Neurotization. The cornea was completely de-epithelized in i) uninjured rats (with normal corneal innervation), ii) denervated rats (with only ophthalmic nerve...
(i) electrocautery, (ii) non-neurotized rats (with CN prior to ophthalmic nerve electrocautery), and (iii) neurotized rats (with CN prior to ophthalmic nerve electrocautery). Six rats were included in each group. Corneal healing was examined 4 weeks after ophthalmic nerve electrocautery to permit reinnervation of the cornea after ophthalmic nerve electrocautery. In the interim, the rat cornea was protected with a permanent tarsorrhaphy. Complete healing of the corneal epithelium occurred between 60 and 84 hours in rats with uninjured (normal) corneal innervation (A). In rats with ophthalmic nerve electrocautery, corneal healing occurred significantly more slowly, and all rats failed to completely heal the corneal epithelium after de-epithelization resulting in significant corneal scarring and corneal perforation in all rats (A). In contrast, corneal reinnervation after corneal neurotization significantly improved corneal healing, with 2 of 6 rats completely healing the cornea and the remaining rats healing significantly more corneal epithelium than denervated animals. Wound size was imaged and calculated every 12 hours after injury up to 96 hours. When wound size was compared over time, corneal healing was significantly improved in rats with corneal neurotization in comparison to rats with ophthalmic nerve electrocautery only (B; * p < 0.01).
4.5 Discussion

Neurotrophic keratopathy (NK) is a major cause of corneal blindness worldwide and it remains one of the more difficult ophthalmologic conditions to treat. The complications of NK are devastating for patients, as persistent corneal epithelial defects and poor healing result in corneal scarring and progressive, permanent vision loss (Lambley et al. 2014; Ramaesh et al. 2007). Conventional treatment for NK, including topical lubricants, tarsorrhaphy and custom protective contact lenses, protect the cornea from injury but fail to prevent the vision loss that occurs in many patients (Sacchetti & Lambiase 2014). Corneal neurotization may effectively treat NK by restoring innervation to the cornea, thereby restoring neurotrophic support and preventing breakdown of the corneal epithelium, subsequent corneal scarring and vision loss.

We developed the first animal model of corneal neurotization using the thy1-GFP+ rat. This model is essential to further investigate whether corneal reinnervation prevents corneal epithelial breakdown and in turn improves healing as a means of preventing long-term scarring of the cornea in NK. The first requirement in investigating corneal reinnervation with donor nerve fibers was a reliable model of NK that resulted in complete denervation of the cornea. We experimented with several models of NK (Ferrari et al. 2011; Nagano et al. 2003; Schimmelpfennig & Beuerman 1982; Wong et al. 2004; Yamaguchi, Turhan, Deshea L. Harris, et al. 2013; Yu & Rosenblatt 2007) in the rat and found that stereotactic electrocautery of the ophthalmic nerve was the most reliable technique to denervate the cornea while leaving the vascular supply to the ocular surface intact (Ferrari et al. 2011; Wong et al. 2004; Nagano et al. 2003). This stereotactic electrocautery resulted in complete corneal denervation one week after injury, but a small amount of corneal reinnervation was apparent after four weeks. To ensure complete corneal denervation when assessing corneal reinnervation four weeks after stereotactic ablation, the procedure was performed a second time one week prior to end-point. Stereotactic ablation of the trigeminal ganglion (TG) was also performed as a means to damage the primary sensory neurons and permanently prevent axon regeneration; however, TG ablation was poorly tolerated with few rats surviving past one week. Poor survival with stereotactic electrocautery of the TG was likely due to the proximity of the TG to the brainstem.

Two procedures of stereotactic electrocautery were required to denervate the cornea for a period of four weeks after corneal neurotization because our preliminary experiments demonstrated that
ablation of the native corneal innervation was essential for reinnervation of the cornea by the donor infraorbital nerve and that the donor nerves required four weeks to reinnervate the cornea after corneal denervation. In contrast, when we performed stereotactic electrocautery of the ophthalmic nerve nine weeks after corneal neurotization, rather than at six weeks, and harvested the cornea one week later, there was insufficient time for donor nerves to reinnervate the cornea. This indicates that corneal denervation is necessary for a novel nerve source to reinnervate the cornea. Four weeks after stereotactic ablation of the ophthalmic nerve, corneal nerve density in rats with corneal neurotization was no different than the uninjured (normal) corneal innervation, however, reinnervation was not as homogenous as the native corneal innervation and the typical whorl-pattern of the sub-basal plexus was not reformed. The significance of this difference requires further investigation.

Corneal reinnervation was achieved in our model by guiding regenerating nerve fibers from the contralateral infraorbital nerve into the cornea via two nerve autografts from the sural and common peroneal nerves, each approximately 30 mm in length, as previously described in patients (Bains et al. n.d.; Elbaz et al. 2014). The surgery of corneal neurotization was performed six weeks prior to stereotactic electrocautery of the ophthalmic nerve because of the long length of the nerve grafts. As nerves regenerate at a rate of approximately 1-3 mm/day, we estimated a 6 week period for regenerating axons from the infraorbital to reach the cornea at which time point we performed the first stereotactic electrocautery procedure in order to denervate the cornea and permit reinnervation by the donor infraorbital nerve.

The contralateral infraorbital nerve was used as a donor because it is more robust in rats than the supraorbital or supratrochlear nerves, and the use of a contralateral nerve allowed for the use of retrograde labeling to confirm the origin of sensory neurons reinnervating the cornea (i.e. as they derive from the contralateral not ipsilateral trigeminal ganglion). Techniques to reinnervate the cornea by directly implanting a transected nerve into the corneal stroma without the use of nerve grafts have been described (Terzis et al. 2009). This was not possible in the rat because the contralateral infraorbital nerve is not long enough to reach the affected, contralateral denervated cornea.

Retrograde labeling of the trigeminal neurons reinnervating the cornea after corneal neurotization confirmed that the entire corneal innervation was derived from the contralateral
The number of neurons labeled was consistent with other published studies, identifying between 50 and 450 neurons innervating the corneal epithelium (De Felipe et al. 1999; Ivanusic et al. 2013; Launay et al. 2015; López de Armentia et al. 2000). Interestingly, the number of trigeminal neurons labeled with retrograde-labeling after corneal neurotization (207 ± 82) was significantly less than the mean (± SD) number of myelinated axons regenerating through the sural and CP nerve graft (8007 ± 1260) identified with histomorphometry. This suggests that the cornea may be regulating either the number or type of nerve fibers that reinnervate the cornea after corneal neurotization. The corneal innervation is composed of a highly regulated network of unmyelinated C fibers and a small number of thinly myelinated (Aδ) fibers that terminate as free-nerve endings in the corneal epithelium (Belmonte 1993; Belmonte & Giraldez 1981; Belmonte et al. 2011). Unlike the corneal innervation, the donor nerves used to reinnervate the cornea with corneal neurotization contain a more diverse population of nerve fibers, including a large number of myelinated fibers. Our model can be used to investigate whether myelinated fibers reinnervate the cornea after corneal neurotization, or whether reinnervation is limited to unmyelinated axons.

In our model of corneal neurotization we demonstrated that corneal reinnervation from the donor infraorbital nerve accelerated healing of the corneal epithelium after wounding the cornea by removing the corneal epithelium. It is likely that nerve fibers reinnervating the cornea restored trophic support to the cornea that is normally supplied by the native corneal innervation. Although this explanation is not exclusive and the molecular mechanisms remain to be investigated. These include investigations of whether 1) nerve fibers contain factors necessary for maintenance of the corneal epithelium, such as Substance P (SP) (Yang et al. 2014; Yamada et al. 2000; Araki-Sasaki et al. 2000; Chikama et al. 1998) or calcitonin gene related peptide,(Mikulec & Tanelian 1996; Tran et al. 2000b) and 2) corneal reinnervation influences proliferation of corneal epithelial or limbal stem cells after injury (Ueno et al. 2012; Yin et al. 2011). Interactions between the corneal epithelium and corneal innervation may also upregulate the expression of α5 integrins and E-cadherin, which are necessary for epithelial adhesion to fibronectin in the extracellular matrix and to maintain the integrity of the corneal epithelium (Nakamura et al. 1998; Chikama et al. 1999; Araki-Sasaki et al. 2000). SP may also play a role in the formation in of corneal epithelial tight junctions by increasing the expression of what is
this ZO-1 (Ko et al. 2009), all of which play a role in supporting the corneal epithelium (Stepp et al. 2016).

This rat model of corneal neurotization can now be used to further investigate corneal neurotization by characterizing the axons that regenerate into the cornea after neurotization and determining how these axons affect the corneal epithelium. These findings will greatly contribute to our understanding of corneal neurotization and support the use of corneal neurotization as a treatment for NK. Surgical reinnervation of the cornea has the potential to completely change the treatment paradigm for these patients by providing a first-line treatment that is capable of preventing corneal epithelial injury and vision loss in patients with NK.
Chapter 5  Improvement of Motor and Sensory Neuron Survival with N-acetyl cysteine after Peripheral Nerve Injury

This chapter is modified from the following:

Catapano J, Zhang JJ, Scholl D, Chiang C, Gordon T, Borschel GH. N-acetyl cysteine prevents retrograde motor neuron death after neonatal peripheral nerve injury. Plastic and Reconstructive Surgery. (Accepted for publication)

5.1 Abstract

**Background:** Nerve transfers require for the donor nerve to be injured, either partially or completely, in order to guide regenerating axons into new targets. Axon regeneration and recovery after nerve grafting and transfer may be limited by retrograde neuronal death after injury. N-acetyl cysteine (NAC) and acetyl-L-carnitine (ALC) improve survival of neurons after adult nerve injury but it is unknown whether they improve survival after paediatric or neonatal injury when neurons are most susceptible to retrograde neuronal death. Our objective is to examine whether NAC or ALC treatment improves survival of neonatal motor or sensory neurons in a rat model of neonatal nerve injury.

**Methods:** Rat pups received either a sciatic nerve crush or transection injury at postnatal day 3 and were then randomized to receive either intraperitoneal vehicle (5% dextrose), NAC (750 mg/kg) or ALC (300 mg/kg) once or twice daily. Four weeks after injury, surviving neurons were retrograde-labeled with 4% Fluorogold. The lumbar spinal cord and L4/L5 dorsal root ganglia were then harvested and sectioned to count surviving motor and sensory neurons.

**Results:** Transection and crush injuries resulted in significant motor and sensory neuron loss, with transection injury resulting in significantly more retrograde neuron death. High-dose NAC (750 mg/kg twice daily) significantly increased motor neuron survival after neonatal sciatic nerve crush and transection injury. Neither NAC nor ALC treatment improved sensory neuron survival.

**Conclusions:** Proximal neonatal nerve injuries produce significant retrograde neuronal death after injury. Treatments improving motor and sensory neuron survival after nerve injury may improve recovery after nerve transfers or nerve grafting.
5.2 Introduction

Motor and sensory reconstruction with nerve grafts and nerve transfers provides a pathway to reinnervate tissue. However, redirecting axons down novel pathways first requires a partial or complete nerve injury in order to perform the coaptation and redirect regenerating axons. Peripheral nerve injuries are known to cause substantial retrograde death of motor and sensory neurons in animal models (Stephen W.P. Kemp et al. 2015; Lowrie & Vrbová 1992), and this may contribute to limiting recovery after nerve repair. Untreated neuronal loss may severely limit the capacity for functional recovery, as the reduced number of neurons cannot fully reinnervate their denervated targets.

Several animal models, including rodents (Stephen W.P. Kemp et al. 2015; Lowrie & Vrbová 1992; Risling, Aldskogius, Hildebrand, et al. 1983; Carlson et al. 1979; Schmalbruch 1984; Schmalbruch 1987b), cats (Risling, Aldskogius & Hildebrand 1983; Carlson et al. 1979), and monkeys (Liss et al. 1996; Liss & Wiberg 1997), have demonstrated that neonatal motor and sensory neurons are profoundly sensitive to retrograde neuronal death following peripheral nerve injury, with loss of up to 80% of neurons (Schmalbruch 1984; Burls et al. 1991; Ekström et al. 1998; Ma et al. 2001; Rossiter et al. 1996). While retrograde neuronal death has not been definitively documented in human neonates, there is recent evidence of retrograde sensory neuron death following distal adult human peripheral nerve injury (West et al. 2013; Suzuki et al. 1993). Neurons are even more susceptible to retrograde neuronal death when injuries are proximal \(^{16,18,19}\), occur at a young age \(^{11,12,17,20}\), and following avulsion injury (Koliatsos et al. 1994; Fu & Gordon 1997; Ygge 1989; W. D. Snider et al. 1992; Li et al. 1998), making it likely that retrograde neuronal death also occurs following more proximal nerve transection injury.

After neonatal peripheral nerve injury, neuronal death is mostly apoptotic (Clowry et al. 1996; Lawson & Lowrie 1998; Bahadori et al. 2001; Chan et al. 2001; Rossiter et al. 1996) and occurs over a period of several days, providing an opportunity to intervene and prevent retrograde neuronal death (Burls et al. 1991; Lowrie et al. 1994). Cell death is triggered by the neurotoxic effects of injury and the loss of neurotrophic support derived from distal targets (Fu & Gordon 1997; Ambron & Walters 1996; Terenghi et al. 2011b). Improving motor and sensory neuron survival after peripheral nerve injury improves functional recovery in a rat model (S.W.P. Kemp et al. 2015). Several agents, including neurotrophic factors (Sendtner et al. 1990; Clatterbuck et
al. 1994; Yuan et al. 2000; Tan et al. 1996; Morcuende et al. 2013; Baumgartner & Shine 1998; Aszmann et al. 2002), NMDA-antagonists (Mentis et al. 1993; Greensmith et al. 1994; Dick et al. 1995; Nógrádi et al. 2007; Petsanis et al. 2012) and inhibitors of apoptosis (Sun et al. 1999; Chan et al. 2001; Chan et al. 2003) have been shown to improve neuronal survival following neonatal nerve injury. However, these compounds are either not approved for clinical use or have intolerable complications that would limit clinical translation.

N-acetyl cysteine (NAC) and acetyl-L-carnitine (ALC) increase the survival of motor and sensory neurons in adult animal models of ventral root rhizotomy and root avulsion (Zhang et al. 2005; Welin et al. 2009; Wilson et al. 2003). Both drugs are also approved for clinical use for other indications. NAC protects the liver in the context of acetaminophen toxicity and ALC is used to promote nerve regeneration in diabetic and chemotherapy-induced neuropathy (De Grandis 2007; Maestri et al. 2005; Chan et al. 2014). Both NAC and ALC have antioxidant properties, and both have been shown to decrease the expression of apoptotic proteins following injury (Reid et al. 2009; Barhwal et al. 2008). Neonatal motor and sensory neurons are significantly more susceptible to retrograde neuronal death than those of adults following peripheral nerve injury (Stephen W.P. Kemp et al. 2015; Lowrie & Vrbová 1992), and therefore, it remains unknown whether NAC and ALC increase motor and sensory neuron survival after neonatal peripheral nerve injury as well as adult nerve injury. If the agents are effective, they could be used clinically to prevent retrograde neuronal death after peripheral nerve injury.

In this study, we determined whether administration of NAC or ALC improves motor and/or sensory neuron survival in a neonatal rat model of peripheral nerve injury. We chose to investigate neuronal survival using both the standard neonatal sciatic nerve crush injury model and a transection injury model. Neonatal crush injury is the standard model for investigating retrograde neuronal survival (Stephen W.P. Kemp et al. 2015; Lowrie & Vrbová 1992); however, transection injury more accurately represents the clinical scenario of many nerve injuries, where meaningful nerve regeneration is not likely to occur without surgical repair (Bade et al. 2014; Chen et al. 2008).
5.3 Materials and Methods

5.3.1 Animals

Pregnant female Lewis rats (E15-18) were obtained from Charles River (Montreal, QC). Prior to study commencement, a statistical power analysis was done using G*Power 3.1 (Faul et al. 2007). Twelve rats were required per surgical paradigm (total of 48 animals amongst the four experiments) to detect a statistically significant difference with a Type I error of 0.05, power 0.9 and an expected effect size of 1.5. Depending on litter size, four to six rats were randomly assigned to each group (total n = 53). Each litter was randomized to include several treatment groups in order to control for variations in litters. At the time of injury, the neonatal rats weighed between 8-10 g. All rats were maintained in a temperature and humidity controlled environment with a 12:12 h light:dark cycle and the mother rats received ad lib water and standard rat chow (Purina, Mississauga, ON). Rat pups were kept with their mothers until study termination.

Surgical procedures were conducted in an aseptic manner with an operating microscope (Leitz, Willowdale, ON). Rats were sacrificed at study termination under deep anesthesia using intraperitoneal (i.p) Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg, Bimeda-MTC, Cambridge, ON). These experiments were approved by The Hospital for Sick Children Laboratory Animal Services (LAS), which adheres to the guidelines of the Canadian Council on Animal Care.

5.3.2 Experimental Design

On post-natal day 3 (P3), neonatal rat pups were divided into one of three sciatic nerve injury groups: i) uninjured, ii) crush, and iii) transection without repair. Sciatic nerve injuries were performed on post-natal day 3 because significant retrograde motor neuron death occurs after neonatal sciatic nerve crush injury prior to post-natal day 5 only (Lowrie et al. 1994; Stephen W.P. Kemp et al. 2015). In each injury paradigm, rats were randomized (n = 53) to receive an i.p. injection of either vehicle (5% dextrose), N-acetyl cysteine (NAC) or acetyl-L-carnitine.
(ALK) immediately after surgery and then daily with a 30G syringe. Four to six neonatal rats were used per group.

In order to compare our results to previous work in our laboratory (S.W.P. Kemp et al. 2015), rats were treated for four weeks, at which point the rats were anesthetized and the sciatic nerve was re-exposed for retrograde-labeling on post-natal day 31 (P31). On post-natal day 34 (P34), three days after retrograde labeling, rats were perfused with 4% paraformaldehyde and the lumbar spinal cord and L4/L5 dorsal root ganglia (DRGs) were harvested, sectioned and the retrograde labeled motor and sensory neurons counted.

5.3.3 NAC and ALC Administration

Following injury, rats were randomized (n = 53) to receive an i.p. injection of either vehicle (5% dextrose), N-acetyl cysteine (NAC) at a dose of 750 mg/kg (Alveda Pharma, Toronto, ON) or acetyl-L-carnitine at a dose of 300 mg/kg (Sigma, Oakville, ON) immediately after surgery and then daily with a 30G syringe (Table 5-1). A separate group of rats were randomized to receive higher dose treatment with twice daily i.p. injections of NAC (750 mg/kg) and ALC (300 mg/kg) to investigate the effects of a two-fold higher dose (Table 5-2). The higher dose of NAC and ALC was administered as a twice daily injection as opposed to a single daily injection because of the limited volume that could be administer i.p per dose in neonatal rats.

The initial daily doses of NAC (750 mg/kg) and ALC (300 mg/kg) were determined based on previous studies in adult rats, demonstrating improved motor and sensory neuron survival with NAC (Hart et al. 2004; Welin et al. 2009) and improved sensory neuron survival with ALC (Karalija et al. 2012; Wilson et al. 2003; Wilson et al. 2007; Hart et al. 2002; Patel et al. 2010) at these doses. Higher dosing of both NAC and ALC was also investigated as neonatal neurons are profoundly more sensitive to retrograde neuronal death than adult neurons11,12,17,20 and therefore it was hypothesized that higher doses may be required.
5.3.4 Surgical model of neonatal peripheral nerve injury

At postnatal day 3 (P3), rats were anesthetized using inhalational anesthetic (2% Isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ). The right sciatic nerve was exposed through a lateral mid-thigh incision. In neonatal rats receiving a sciatic nerve crush injury, the sciatic nerve was crushed with a standard #5 jeweler’s forceps for 30 s at the lower border of the quadratus femoris muscle (Figure 5-1A). As previously described (Stephen W.P. Kemp et al. 2015; S.W.P. Kemp et al. 2015), injuries were made in relation to the quadratus femoris muscle as this landmark was found to be more reliably discernable in neonatal rats than the trifurication of the sciatic nerve. Complete sciatic nerve crush was confirmed by observing translucency in the section of nerve at the location of the crush.

For neonatal rats in which the sciatic nerve was transected, a 9-0 Nylon suture was tied around the sciatic nerve at the level of the quadratus femoris muscle and the nerve was then transected immediately distal to the suture (Figure 5-1B). The distal portion of the sciatic nerve was then removed to the level of the knee to ensure that regrowth of the transected nerve would not occur. Following surgery, all rat pups were returned to their home cages with their mothers, and were dosed for four weeks with either vehicle, NAC, or ALC as described previously. The pups received Metacam (0.3 mL/100 g body weight; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) for post-operative pain relief.

Figure 5–1 Model of Neonatal Crush and Transection Injury. The sciatic nerve was exposed through a midlateral incision and either crushed (A) or transected (B) distal to the quadratus femoris muscle. To prevent nerve regeneration after sciatic nerve transection, a suture (9-0 Nylon) was tied around the proximal transected stump and a portion of the distal nerve was excised.
5.3.5 Retrograde-labeling of surviving neurons

Retrograde labeling was performed four weeks following the initial surgery (at P31) to identify and count the surviving motor and sensory neurons (Catapano et al. 2016). A four week time point was used in order to compare our results to previous results obtained in our lab with another neuroprotective pharmaceutical (S.W.P. Kemp et al. 2015). The right sciatic nerve was re-exposed through a mid-lateral thigh incision and transected 5 mm proximal to either the original crush site or the site of the nerve transection and ligation. To construct the petrolatum well, we first placed the petrolatum in a 1 mL syringe with a 25 gauge half-inch needle, which permitted accurate distribution of the petrolatum. The well was then constructed by placing two layers of petrolatum around the periphery of a small piece of plastic paraffin film (approximately 0.5 mm x 0.5 mm) adjacent to the transected nerve tip while ensuring that the nerve does not come in contact with the petrolatum as this may decrease labeling by preventing dye uptake. The proximal nerve stump was then carefully draped over the well so that the nerve tip was centered within the well and another layer of petrolatum was placed over the existing well walls to ensure no leakage occurred around the nerve. It was necessary to ensure that while the petrolatum was being placed that it came in contact with the Parafilm and nerve stump, otherwise leakage of the well occurred. The well was then filled with 10 µL of 4% solution of FluoroGold (FG: Fluorochrome LLC, Denver, CO) dissolved in sterile saline solution and the nerve was left within the well for one hour, after which the well was removed and staining was confirmed visually by observing yellow staining of the exposed nerve tip. The proximal stump and wound were then rinsed three times with sterile saline to remove any residual dye and prevent staining of the adjacent muscle or nerve. Incisions were closed and, following recovery, animals were returned to their cages.

5.3.6 Counting of motor and sensory neurons

Three days following retrograde labeling on post-natal day 34 (P34), rats were euthanized using intraperitoneal Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg,Bimed-MTC, Cambridge, ON) and perfused with 4% paraformaldehyde (PFA). The lumbar spinal cord
and L4 and L5 DRGs were harvested and post-fixed in 4% PFA for one day and then cryoprotected in 30% sucrose in 4% PFA for four days prior to embedding in optimal cutting temperature compound (OCT: Sakura Fine Technical Co., Torrence, CA). Spinal cords and DRGs were serially sectioned at 30 µm and 20 µm respectively, using a cryostat (Leica Microsystems Inc., Concord, ON) at -22°C and mounted onto Superfrost slides (Fisher Scientific, Ottawa, ON). Retrograde labeled motor and sensory neurons in each spinal cord section and every fifth DRG section respectively, were counted on a fluorescent microscope with a 10x objective (100x overall magnification; Leica). A blinded observer performed all counts. Correction for double counting was made using a correction factor, calculated as 0.48 by our laboratory using the method previously described (Abercrombie 1946).

5.3.7 Statistical analysis

All statistical analysis was performed using GraphPad Prism® version 6.0 for Mac (GraphPad Software, Inc; San Diego, California). All data was analyzed using a one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction applied, except for data in Figure 3A and 4C which was analyzed with a Student’s t-test as animals treated with NAC were compared only with vehicle treated animals. Statistical significance was accepted at the level of $p < 0.05$; all data are expressed as the mean ± SD.
5.4 Results

5.4.1 Neonatal nerve injury results in significant motor and sensory neuron loss

Retrograde labeling of the sciatic nerve in uninjured rats four weeks after birth identified $1160 \pm 57$ motor neurons and $4731 \pm 218$ sensory neurons in the rat lumbar spinal cord and L4 and L5 DRG respectively. Both sciatic nerve crush and transection injuries three days after birth resulted in significant reduction in the numbers of surviving motor and sensory neurons (Figure 5-2). Retrograde labeling 5 mm proximal to site of nerve injury four weeks after sciatic nerve crush injury demonstrated a 64% and a 48% reduction in the number of surviving motor and sensory neurons, respectively ($p < 0.001$). A neonatal sciatic nerve transection injury resulted in even greater neuronal loss, with retrograde labeling of the neurons identifying only 11% and 36% of motor and sensory neurons, respectively ($p < 0.001$).
Figure 5–2 Neuron Survival After Neonatal Nerve Injury. There was a significant reduction in the numbers of (A) motor and (B) sensory neurons four weeks after both sciatic nerve crush and transection injuries in comparison to the uninjured sciatic nerve (\( ** p < 0.001 \)). Representative images of retrograde labeled motor neurons, in 50 µm longitudinal lumbosacral spinal cord sections, are shown for (C) uninjured rat pups, and after (D) sciatic nerve crush, and (E) sciatic nerve transection injuries (bar is equal to 250 µm). The number of rats in each group and motor and sensory neuron counts are summarized in Table 5-1 and 5-2.
5.4.2 High-dose NAC significantly increases motor neuron survival after neonatal crush injury

Intraperitoneal (i.p) injection of NAC (1500 mg/kg) significantly improved motor neuron survival following neonatal crush injury in comparison to animals treated with vehicle or ALC ($p = .003$) (Table 5-1 and Fig. 5-3A). Treatment with a lower dose of NAC, with once daily i.p injection of NAC (750 mg/kg), had no significant effect on numbers of motor neurons that survived after neonatal sciatic nerve crush injury (Figure 5-3B). Treatment with i.p. injections of ALC (300 mg/kg) either once or twice daily also failed to promote motor neuron survival following crush injury (Figure 5-3A/B).

![Figure 5–3 Treatment Twice Daily with NAC Increases Motor Neuron Survival following Nerve Crush.](image)

(A) Intraperitoneal injection of NAC (750 mg/kg) twice daily following crush injury significantly increased motor neuron survival. (* $p < 0.05$) (B) Decreasing administration of NAC to once daily injection and treatment with ALC at either dose did not affect motor neuron survival. The number of animals in each group and motor and sensory neuron counts are summarized in Table 5-1 and 5-2.

5.4.3 Treatment with high-dose NAC significantly increases motor neuron survival after neonatal transection injury

Intraperitoneal (i.p) injection of NAC (1500 mg/kg) daily significantly improved motor neuron survival in comparison to vehicle treated animals following neonatal transection injury ($p = 0.03$)
Once daily i.p. injection of NAC (750 mg/kg) did not significantly improve motor neuron survival following neonatal sciatic nerve transection injury (Figure 5-4B) and likewise, once daily i.p. injection of ALC (300 mg/kg) did not improve motor neuron survival (Fig 5-4B). In the transection injury model, the effect of twice daily injections of ALC on motor neuron survival was not investigated, as the same ALC dose had no effect on motor neuron survival after a less severe crush injury.

![Figure 5-4 Motor Neuron Survival is Improved Following Transection injury with NAC.](image)

(A) Treatment with NAC (750 mg/kg) twice daily increased motor neuron survival following sciatic nerve transection injury. (B) Decreasing administration of NAC to once daily injection and treatment with ALC at either dose did not increase motor neuron survival. The number of animals in each group and motor and sensory neuron counts are summarized in Table 5-1 and 5-2.

### 5.4.4 NAC and ALC do not improve sensory neuron survival after crush or transection injury

Following either neonatal sciatic nerve crush or transection injury, daily i.p. injection of either NAC (750 mg/kg) or ALC (300 mg/kg) did not affect sensory neuron survival in comparison to rats treated with vehicle (Figure 5-5A/B). Increasing the daily dose of NAC, with i.p. injection of rats twice daily with NAC (1500 mg/kg) also failed to improve sensory neuron survival following neonatal sciatic crush or transection injury (Figure 5-5C/D). As for motor neuron survival in the crush injury model, the twice daily injections of ALC were not carried out because the twice daily injection of ALC (300 mg/kg) had no effect on the survival of sensory neurons after the less severe sciatic nerve crush injury.
Figure 5–5 Neither NAC nor ALC Improve Sensory Neuron Survival. Following neonatal sciatic nerve crush injury, neither NAC nor ALC improved sensory neuron survival when rats were treated with i.p. injections either (A) twice daily or (B) daily. While transection injury results in more retrograde sensory neuron death, neither NAC nor ALC improved sensory neuron survival when rats were treated either (C) twice daily or (D) daily. The number of animals in each group and motor and sensory neuron counts are summarized in Table 5-1 and 5-2.
### Table 5–1 Motor and Sensory Neuron Survival After Neonatal Sciatic Nerve Crush Injury.

Values are mean ± STD. The number in each group is given in parenthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of MNs</th>
<th>% Survival of MN</th>
<th>No. of SNs</th>
<th>% Survival of SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured</td>
<td>1160 ± 57 (6)</td>
<td></td>
<td>4731 ± 218 (6)</td>
<td></td>
</tr>
<tr>
<td>Crush + dextrose</td>
<td>422 ± 144 (6)</td>
<td>36 ± 12.4</td>
<td>2463 ± 140 (6)</td>
<td>52 ± 3.0</td>
</tr>
<tr>
<td>Crush + ALC (300 mg/day)</td>
<td>467 ± 112 (6)</td>
<td>40 ± 9.7</td>
<td>2681 ± 181 (6)</td>
<td>57 ± 3.8</td>
</tr>
<tr>
<td>Crush + ALC (600 mg/day)</td>
<td>446 ± 121 (4)</td>
<td>38 ± 10.4</td>
<td>2441 ± 117 (4)</td>
<td>52 ± 2.5</td>
</tr>
<tr>
<td>Crush + NAC (750 mg/day)</td>
<td>494 ± 38 (6)</td>
<td>43 ± 3.3</td>
<td>2542 ± 305 (6)</td>
<td>54 ± 6.4</td>
</tr>
<tr>
<td>Crush + NAC (1500 mg/day)</td>
<td>755 ± 63 (4)</td>
<td>65 ± 5.4</td>
<td>2658 ± 370 (4)</td>
<td>56 ± 7.8</td>
</tr>
</tbody>
</table>

### Table 5–2 Motor and Sensory Neuron Survival After Neonatal Sciatic Nerve Transection.

Values are mean ± STD. The number in each group is given in parenthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of MNs</th>
<th>% Survival of MN</th>
<th>No. of SNs</th>
<th>% Survival of SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured</td>
<td>1160 ± 57 (6)</td>
<td></td>
<td>4731 ± 218 (6)</td>
<td></td>
</tr>
<tr>
<td>Trans. + dextrose</td>
<td>132 ± 53 (6)</td>
<td>11 ± 4.6</td>
<td>1704 ± 200 (6)</td>
<td>36 ± 4.2</td>
</tr>
<tr>
<td>Trans. + ALC (300 mg/day)</td>
<td>129 ± 80 (5)</td>
<td>11 ± 6.9</td>
<td>1889 ± 524 (5)</td>
<td>40 ± 11.1</td>
</tr>
<tr>
<td>Trans. + NAC (750 mg/day)</td>
<td>150 ± 39 (6)</td>
<td>13 ± 3.4</td>
<td>2088 ± 468 (6)</td>
<td>44 ± 9.9</td>
</tr>
<tr>
<td>Trans. + NAC (1500 mg/day)</td>
<td>241 ± 65 (4)</td>
<td>21 ± 5.6</td>
<td>1794 ± 287 (4)</td>
<td>38 ± 6.1</td>
</tr>
</tbody>
</table>
5.4.5 High-dose NAC resulted in significant weight loss

Rat pups treated with high-dose NAC gained significantly less weight by the time of sacrifice at post-natal day 33 than litter mates treated with vehicle (80 g ± 5 vs 94 ± 9; p < 0.001) (Figure 5-6A). Differences in weight were greatest at 34 days and most apparent when rats were separated into subgroups of male and female rat pups (Figure 5-6B). When analysed separately, male rats treated with high-dose NAC gained approximately 22 g less of weight in comparison to littermates treated with vehicle by the date of sacrifice (P34) (83 g ± 5 vs 104 ± 3; p < 0.001). Likewise, female rats treated with high-dose NAC also gained approximately 12 g less weight than littermates treated with vehicle by the date of sacrifice (P34) (77 g ± 4 vs 89 ± 4; p < 0.001). Rat pups treated with lower doses of NAC or ALC exhibited no differences in weight gain in comparison to littermates treated with vehicle (data not shown). Through observation, all rats treated with high-dose NAC also appeared more lethargic and relatively underdeveloped in comparison to littermates although this difference was not investigated further or quantified.

Figure 5–6 Animals Treated with High-dose NAC Gained Less Weight. After both crush and transection neonatal nerve injury, rat pups treated with high-dose NAC demonstrated less weight gain than littermates treated with vehicle. These differences were statistically different over time when compared with repeated measures ANOVA (p < 0.001). When analyzed separately as male and female subgroups, both male and female rats were significantly lighter at the date of sacrifice (P 34) in comparison to vehicle treated littermates (p < 0.001).
5.5 Discussion

We found that high-dose N-acetyl cysteine (NAC), when administered twice daily, significantly increased motor neuron survival after both sciatic nerve crush and transection injuries in neonatal rats. This is consistent with findings of improved motor neuron survival with NAC treatment in adult models of peripheral nerve injury (Zhang et al. 2005; Karalija et al. 2012). However, much higher doses (1500 mg/kg/d as compared to 750mg/kg/d) were required to protect injured motor neurons in neonatal rats in our study. In contrast, we also found that both NAC and acetyl-L-carnitine (ALC) were ineffective at reducing sensory neuron death after neonatal peripheral nerve injury.

This contrasts previous work in adult models of peripheral nerve injury, where both NAC (Hart et al. 2004; Welin et al. 2009) and ALC (Karalija et al. 2012; Wilson et al. 2003; Wilson et al. 2007; Hart et al. 2002) improved sensory neuron survival and once-daily dosing of NAC improved motor neuron survival (Zhang et al. 2005). Neonatal neurons are profoundly more sensitive to retrograde neuronal death than adult neurons\(^{11,12,17,20}\), and, therefore, higher doses of NAC and ALC may be required to improve motor and sensory neuron survival after neonatal nerve injuries. In adult models of peripheral nerve injury, higher doses NAC are more effective at improving motor neuron survival after ventral rhizotomy and avulsion injuries, and the same has been shown for ALC, with higher treatment doses demonstrating more effective protection of adult sensory neurons (Zhang et al. 2005; Wilson et al. 2003). In our study, NAC treatment after neonatal peripheral nerve injury improved only motor neuron survival and not sensory neuron survival, which may be due to differences in sensitivity of motor and sensory neurons to retrograde neuronal death after injury (Lewis et al. 1999; Benn et al. 2002).

Retrograde neuronal death after peripheral nerve injury is mainly apoptotic in nature, and NAC may improve motor neuron survival by inhibiting injury-induced neuronal apoptosis. While apoptosis is crucial for the normal development of the nervous system, it is inappropriately triggered after peripheral nerve injury due to the neurotoxic effects of injury and the loss of neurotrophic support derived from distal targets (Fu & Gordon 1997; Ambron & Walters 1996; Terenghi et al. 2011b). Injured motor neurons exhibit oxidative stress after injury and studies have shown that apoptosis is dependent on the pro-apoptotic protein Bax (Deckwerth et al. 1996; Martin & Liu 2002; Reid et al. 2009). As a known antioxidant, NAC may decrease apoptosis by
decreasing oxidative stress on the cell (Yan & Greene 1998; Chan et al. 2014) and NAC down-regulates expression of Bax (Reid et al. 2009). Neonatal motor and sensory neurons may experience greater oxidative stress than adult neurons after injury, requiring larger doses of NAC to prevent retrograde neuronal apoptosis after neonatal nerve injury. This is consistent with previous findings of improved neuronal survival after adult peripheral nerve injury, where NAC treatment was less effective in more severe injury paradigms (Zhang et al. 2005).

While we found that only higher dose NAC (1500 mg/kg/d) reduced retrograde motor neuron death, it remains possible that treatment efficacy with 1500 mg/kg/d was not due to the increased overall dose but instead due to the altered administration schedule, as the half-life of NAC is known to be approximately 2.5 hours in humans (Borgström et al. 1986). Due to limitations in the amount of NAC that could be administered in one injection, the higher dose was administered as two injections of 750 mg/kg in the morning and evening, rather than a once daily injection. Further investigation is required to discern whether the higher dose NAC was effective because of the higher overall daily dose of NAC or because it was administered twice daily rather than once daily as with the lower (750 mg/kg) dose. More frequent dosing may limit the overall daily dose required to prevent retrograde neuronal death and potentially also limit systemic toxicity.

Neonatal sciatic nerve crush injury is an established model for investigating retrograde motor and sensory neuron death in neonatal rats (Bridge et al. 1994; Stephen W.P. Kemp et al. 2015; Lowrie & Vrbová 1992). Nerve crush injury (Sunderland type II) causes minimal endoneurial tube damage but supports significant nerve regeneration and recovery. In contrast, peripheral nerve transection injuries (OBPI) often consists of Type IV and V nerve injuries, which require surgical intervention to remove scar tissue and realign the proximal and distal nerve ends. A neonatal sciatic nerve transection models peripheral nerve transection more accurately as it prevents nerve regeneration into the distal stump, resulting in significantly more neuron loss after neonatal peripheral nerve injury (Kashihara et al. 1987; Ma et al. 2003).

In addition to NAC, several other agents have also demonstrated improvement in motor neuron survival following neonatal peripheral nerve injury. These include inhibitors of glutamate-induced excitotoxicity (Mentis et al. 1993; Greensmith et al. 1994; Dick et al. 1995; Nógrádi et al. 2007; Petsanis et al. 2012), neurotrophic factors (Sendtner et al. 1990; Clatterbuck et al. 1994;

While several of these agents are more potent than NAC in protecting neonatal motor neurons from neuronal death, none of these agents are approved for use in humans, in part because possible adverse effects on development have not been determined.

While N-acetyl cysteine has a long history of safe use clinically in patients with cystic fibrosis and acetaminophen toxicity, we found that treatment with high-dose NAC (1500 mg/kg/day) resulted in significantly less weight gain than neonatal rat pups treated with low-dose NAC (750 mg/kg/day), ALC or vehicle. NAC (1500 mg/kg/day) treated rat pups also appeared more lethargic when examined. For acetaminophen toxicity, N-acetyl cysteine is used at much lower doses (300 mg/kg) for a shorter period of time (24 hours) and is known to result in anaphylactoid reactions. Severe systemic adverse reactions have been also been reported with NAC following iatrogenic overdose (Mahmoudi et al. 2015; Sandilands & Bateman 2009). Toxicity in rats with high-dose NAC has been shown in other studies, however the mechanism remains unknown (Sprong et al. 1998). Animal studies have suggested that high-doses (1200 mg/kg/day) may decrease hepatic glucose metabolism and impair liver regeneration, which both may adversely affect growth (Yang et al. 2009; Zwingmann & Bilodeau 2006). Given these findings, further investigation into the side effects of long-term high-dose NAC treatment is required prior to the clinical translation of these results.

Given recent findings in adults (West et al. 2013; Suzuki et al. 1993), it is highly likely that retrograde death of motor and sensory neurons is an overlooked component of disability following peripheral nerve injury. Neuronal death may limit functional recovery following nerve injuries and surgical repair. N-acetyl cysteine (NAC) is an attractive agent for clinical translation as it is already approved for clinical use in neonates for other indications. However, further investigation into other treatment strategies, such as localized drug delivery systems or more frequent dosing regimens, is required. This may limit systemic toxicity of NAC while still providing motor neurons with high enough doses to prevent retrograde neuronal death (Wood et al. 2013; Zhang et al. 2005).
Chapter 6  Concluding Summary, General Discussion, and Future Directions
6.1 Concluding Summary

Corneal neurotization reinnervates the cornea using functioning sensory nerves from elsewhere on the face. As such, corneal neurotization is the only treatment for neurotrophic keratopathy (NK) that addresses the underlying lack of corneal reinnervation that causes NK. In order for corneal neurotization to be adopted as part of the treatment algorithm for NK, research is needed to demonstrate that after corneal neurotization foreign donor axons:

i. Reinnervate the cornea
ii. Restore corneal sensation
iii. Improve ocular surface health (i.e. improve maintenance of the corneal epithelium and improved corneal epithelial healing after injury)

In a prospective clinical research study (Chapter 3), we demonstrated using in vivo corneal confocal microscopy (IVCCM), immunohistochemistry (IHC) and magnetoencephalography (MEG) that axons reinnervate the cornea after corneal neurotization. With MEG, we also demonstrated that the evoked response with stimulation of the neurotrophic cornea after corneal neurotization derived from the ipsilateral somatosensory cortex after surgery; a location consistent with the contralateral donor supratrochlear nerve used to reinnervate the cornea in that patient. Corneal reinnervation also significantly improved corneal sensation when tested with Cochet-Bonnet esthesiometry. This data demonstrates convincingly the corneal neurotization reinnervates the cornea and restores corneal sensation.

We were able to show stabilization of visual acuity in these patients, with no further vision loss associated with corneal neurotization or NK in 17 of 19 eyes. Significant improvements in vision would not be expected in these patients because of extensive pre-existing scarring of the corneal stroma due to longstanding NK. Corneal transplantation is the only method of resolving extensive stromal scarring in patients with advanced NK, however corneal transplantation for visual rehabilitation is normally contraindicated in patients with NK because of subsequent failure and scarring of the graft in the absence of corneal innervation. Due to significant improvements in corneal sensation and documentation of corneal reinnervation after corneal neurotization, the ophthalmologists at The Hospital for Sick Children felt comfortable offering corneal transplants to three patients to improve visual acuity. Two of the corneal transplants remained clear with over two years of follow-up, suggesting that corneal neurotization also
improves corneal epithelial maintenance and healing. Improved maintenance and healing of the corneal epithelium was also suggested by a decrease in the number of persistent epithelial defects patients experienced after corneal neurotization. However, this study was not randomized and patients received other ophthalmic treatments for NK in addition to corneal neurotization. While most patients had a de-escalation of other ophthalmic treatments following corneal neurotization, further investigation is required to document whether corneal neurotization does in fact improve ocular surface health. Improving upon our prospective cohort trial by further investigating corneal neurotization clinically through a randomized clinical trial is difficult because of the heterogeneity and rarity of NK. Furthermore, due to the poor outcomes expected with NK, clinical equipoise does not exist to justify a randomized trial restricting access to corneal neurotization for some patients.

Therefore, to complement our clinical research and further investigate corneal neurotization, we developed an animal model of NK (Chapter 3) and corneal neurotization (Chapter 4). Development of the animal model required a significant amount of investigation to produce a model of NK that resulted in prolong corneal denervation in order to permit corneal reinnervation from regenerating axons in the donor nerve grafts. Using the thy1-GFP+ rat, we demonstrated that donor axons from the contralateral infraorbital nerve were capable of reinnervating the cornea and restoring corneal axon density, confirming our clinical findings. Interestingly, a small proportion of regenerating neurons reinnervated the cornea, suggesting that the cornea may be regulating axon regrowth after corneal neurotization. Most importantly, we then used this model to demonstrate that corneal neurotization improves corneal epithelial healing after injury in rats with NK. These findings suggest that axons reinnervating the cornea from a foreign donor nerve also restore the cornea with neuromediators necessary to promote corneal healing after injury. Using our model of corneal neurotization, further investigation is required to investigate how corneal neurotization improves corneal epithelial healing after injury and whether this leads to decreased corneal scarring and loss of visual acuity over time.

While results after corneal neurotization have been impressive, it is important to recognize that sensory and motor recovery with nerve grafts and transfers is limited by poor axon regeneration. Aside from microsurgical techniques, minimal progress has been made in improving axon regeneration after peripheral nerve repair. An approach to improving peripheral nerve repair must be multimodal in order to overcome the large number of challenges that exist for
regenerating peripheral axons. In Chapter 6, we investigated a pharmacologic means of reducing retrograde motor and sensory neuron death after peripheral nerve injury. Here we showed, that in a neonatal model, where motor and sensory axons are most susceptible to retrograde neuronal death, treatment with a high-dose of N-acetyl cysteine improves motor neuron but not sensory neuron survival. Pharmacologic rescue of motor and sensory neurons may in the future play a role in improving recovery after peripheral nerve transection and repair.
6.2 General Discussion

Corneal neurotization is a novel surgical procedure that provides hope for patients with neurotrophic keratopathy (NK). Conventional ophthalmic management for NK is designed to manage the complications of the disease, but fails to address the underlying cause of impaired corneal sensation. As such, conventional treatment fails to corneal epithelial breakdown and ulceration in patients with NK and many progress to develop significant vision loss and blindness. Conventional NK treatment consists of lubricating ophthalmic drops to protect the corneal epithelium from ulceration. If these are ineffective, patients depend on other means to protect the corneal surface from injury, including a corneal or scleral lenses or surgical tarsorrhaphy that sutures the eyelids either partially or completely closed. These treatments are disfiguring and impede vision. Alternative and better solutions are needed for these patients.

Recent developments have been made to accelerate healing of persistent epithelial defects (PEDs) in patients with NK. These topical ophthalmic pharmacological agents aim to restore the cornea with trophic mediators that improve corneal healing and include topical Substance P (Yamada et al. 2008), NGF (Lambiase et al. 1998; Ferrari et al. 2014), autologous serum and platelet rich plasma (Sacchetti & Lambiase 2014; Soni & Jeng 2016; Khokhar et al. 2005). These treatments, however, do not correct the underlying abnormality in NK and are targeted to improve corneal healing after the development of a persistent corneal ulceration. Therefore, they fail to prevent the major complication of NK (i.e. breakdown of the corneal epithelium) and, therefore, while they may improve healing of the corneal epithelium they may not significantly reduce vision loss in these patients over time. Furthermore, these topical pharmacologic agents are extremely expensive and patients remain dependent on daily treatment and regular physician follow-up to diagnose the corneal epithelial breakdown and injury, which the patients are unable to detect independently in the absence of corneal sensation.

Corneal neurotization addresses the underlying pathophysiology of NK by reinnervating the cornea with axons from functioning donor nerves from elsewhere on the face. This surgical approach aims to prevent rather than manage the complications of NK by restoring corneal innervation and therefore may be more successful at preventing long-term vision loss. Previous surgical techniques of corneal neurotization have been described in the literature, but these were excessively invasive and, as a result, were not adopted elsewhere to treat patients with NK
(Samii 1981; Terzis et al. 2009). The technique of corneal neurotization investigated in this thesis, used two minimally invasive incisions above the orbit and a nerve autograft to guide regenerating axons from the donor nerve into the neurotrophic cornea. This mitigates the need for a frontoorbital craniotomy or bicoronal incision, making this a surgical solution more palatable for surgeons and patients. However, in order for corneal neurotization to be adopted as part of the treatment algorithm for NK, further research is required to demonstrate definitively that corneal neurotization improves maintenance and repair of the corneal epithelium, and in turn, prevents the long-term complication of vision loss in patients with NK.

In our prospective cohort trial, we demonstrated that corneal neurotization reinnervates the cornea in patients with NK and improves corneal sensation. Due to developmental delay and other ocular comorbidities, a small number of patients were eligible for \textit{in vivo} corneal confocal microscopy (IVCCM) and magnetoencephalography (MEG). MEG and IVCCM required that patients remain still for long periods of time and follow instructions, which was not possible for the majority of our patients that were pediatric. IHC could only be performed in the three patients undergoing corneal transplantation. Despite the small number of patients included in the documentation of corneal reinnervation, IVCCM, MEG and IHC all provided evidence of corneal reinnervation after corneal neurotization. In conjunction with the corneal sensory data, these results are convincing that donor axons reinnervate the cornea after corneal neurotization and restore corneal sensation.

Importantly, we also demonstrated that no further loss in visual acuity occurred in 17 of 19 treated eyes in the patients that we studied with NK. Vision would not be expected to improve significantly in this patient population as most patients had extensive preexisting corneal scarring that is only correctable with corneal transplantation. We also provided evidence that corneal neurotization improves ocular surface health. Despite fewer topical ophthalmic agents being used in most patients and reversal of tarsorrhaphy in three patients, fewer persistent epithelial defects (PEDs) were experienced after corneal neurotization and two patients underwent corneal transplantations that remain clear two years post-transplant. This contrasts the success of corneal transplantation in this same population prior to corneal neurotization. In the four patients that received corneal transplantation prior to corneal neurotization, on an emergency basis to repair the integrity of the ocular surface because of extensive corneal perforations, all grafts subsequently became scarred and opacified secondary to NK. We were unable to demonstrate
that this difference in outcomes of corneal transplantation was statistically significant because of small patient numbers, however the result is clinically significant as it is well-established that corneal transplantation is relatively contraindicated for visual rehabilitation in patients with NK because the graft subsequently becomes scarred quickly in the absence of corneal sensation. Corneal neurotization may provide an opportunity for patients with significant corneal scarring from NK to regain their sight by reinnervating the cornea and permitting successful corneal transplantation. As corneal neurotization is adopted as an established treatment for NK, the goal would be to investigate corneal neurotization as a first-line treatment early in the course of the disease to prevent corneal scarring in patients with NK.

In order to be adopted widely as a treatment for NK, it is necessary to document definitively that corneal neurotization improves ocular surface health. The gold standard in determining this clinically would be a RCT. RCTs are difficult in this patient population however, due to the rarity and heterogeneity of NK, making it difficult to recruit enough patients and to demonstrate statistical differences. Furthermore, due to the success of corneal neurotization in our prospective cohort trial and the lack of adequate alternative treatments, equipoise does not exist to justify a RCT. Clinical research is limited by the questions that can be addressed and answered by further investigation. Characterizing nerve phenotype after corneal reinnervation and investigating whether corneal reinnervation improves corneal epithelial healing requires an animal model in which tissue can be harvested and analyzed. For these reasons, we developed a novel rat model of NK and corneal neurotization to complement our clinical research. We designed these models in the rat because rodent research is relatively less expensive and rats demonstrate robust axon regeneration that significantly reduces the amount of time required to do the experiments in comparison to larger animal models such as rabbits and monkeys.

Using our model of corneal neurotization, we demonstrated that donor axons from the contralateral infraorbital nerve reinnervate the cornea in rats with NK, confirming our clinical findings. A small number of neurons (~ 200) were found to reinnervate the cornea in comparison to the large number of myelinated axons that regenerated through the nerve autografts (~ 9000), suggesting that the cornea may somehow be regulating reinnervation after corneal neurotization. After describing our model of corneal neurotization, we demonstrated that axons reinnervating the cornea after corneal neurotization significantly improve corneal healing in rats with NK. This result suggests that axons reinnervating the cornea after corneal neurotization contain
neuromediators, such as Substance P, that are necessary for corneal epithelial healing after injury (Müller et al. 2003; Shaheen et al. 2014). Documentation of improved healing after corneal neurotization suggests these axons may also contain neuromediators that maintain the corneal epithelium and prevent breakdown, preventing corneal ulcerations and subsequent corneal scarring that progresses to vision loss in patients with NK. These findings must be further investigated by determining the phenotype of axons reinnervating the cornea after corneal neurotization and how corneal neurotization affects the corneal epithelium in the absence of corneal epithelial injury.

In Chapter 5, we investigated a pharmacologic means of decreasing retrograde neuronal death after peripheral nerve injury. In order to perform the nerve transfers that are used for corneal neurotization, the donor nerve must first be transected prior to coaptation to the nerve graft (Sunderland V injury). Sensory neuron death after adult peripheral nerve injury has been documented in human patients with MRI imaging (West et al. 2013; Suzuki et al. 1993). Neurons are most susceptible to retrograde neuronal death when injuries are close to their cell bodies (proximal nerve injuries) (Hu & McLachlan 2003; Ma et al. 2001; Terenghi 1999), occur at a young age (Burls et al. 1991), and with increasing injury severity (Koliatsos et al. 1994; Fu & Gordon 1997; Ygge 1989; W. D. Snider et al. 1992; Li et al. 1998), making it likely that retrograde neuronal death also occurs when performing nerve transfers in pediatric patients. Sensory and motor functional recovery are dependent on neuron survival and axon regeneration across the nerve grafts. We investigated whether two pharmacologic agents approved for clinical use for other indications, N-acetyl cysteine (NAC) and acetyl-L-carnitine (ALC), which had demonstrated motor and sensory neuron survival in adult rat studies of peripheral nerve injury, (Zhang et al. 2005; Welin et al. 2009; Wilson et al. 2003) would reduce retrograde neuronal death in a neonatal model of peripheral nerve injury. In the neonatal rat model of peripheral nerve injury, we found that ALC did not rescue either motor or sensory neurons from neuronal cell death but that NAC rescued a small proportion of motor neurons after injury. Extremely high doses of NAC were needed in order to see an effect, which resulted in poor weight gain in treated rats, making clinical translation of these results with the current dosing schedule impossible. It is possible that more frequent dosing of NAC could have a dose limiting effect, however this question requires further investigation.
Although we have demonstrated convincingly that corneal neurotization reinnervates the cornea and improves healing after corneal ulceration, it is not expected that corneal neurotization will be a panacea for these patients. Regardless of the etiology of corneal sensory impairment, patients often have concomitant lacrimal gland or facial nerve (CN VII) palsy, which leaves patients susceptible to dry eye disease and exposure keratopathy. Despite this, corneal neurotization addresses the neurotrophic component of keratopathy in these patients and appears to improve ocular surface health and prevent corneal injury. It significantly improves the disease state and also empowers patients by providing them with corneal sensation and a means of detecting corneal injury, allowing them to seek medical attention if required.
### 6.3 Future Directions

Reinnervating the cornea using donor nerves is a novel concept and, while there exist some clinical publications that include a small number of patients (Terzis et al. 2009; Samii 1981; Elbaz et al. 2014; Jacinto et al. 2016) there is no basic science literature that concerns reinnervation of the cornea from donor nerves. Previous animal studies have been limited to investigating nerve regeneration into the cornea in animal models of corneal disease including HSV-1 keratitis (Martin et al. 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 transplantation (Omoto et al. 2012), and corneal abrasion (Li et al. 2011). These models investigate regeneration of the corneal innervation after the corneal nerves have been damaged within the cornea itself. This is a very different research question, as this investigates regeneration of the normal corneal innervation, which would be expected to contain the neuromediators necessary to promote maintenance and healing of the corneal epithelium. No previous publications investigate the influence of foreign donor nerves reinnervating the cornea, as is the case with corneal neurotization. However, due to the severity of neurotrophic keratopathy (NK) and the management dilemma it poses for physicians, an extensive amount of research has been conducted that characterizes the normal corneal innervation and investigates how corneal denervation influences the corneal epithelium. Based on this background, we have the following future directions to investigate corneal reinnervation after corneal neurotization.

**Future Direction # 1:** A necessary step for the further the investigation of corneal neurotization is to characterize the nerve fibers that reinnervate the cornea after corneal reinnervation and to investigate whether nerve graft-derived Schwann cells (SCs) migrate into the cornea to support axon regeneration. The rationale for this is that the majority of the corneal nerves are unmyelinated while the donor nerve contains a diverse population of myelinated and unmyelinated axons that normally innervate skin. Understanding the type of nerve fibers that reinnervate the cornea after corneal neurotization would help elucidate whether the donor nerve determines the types of nerve fibers that reinnervate the cornea or whether the corneal milieu selects for the types of nerve fibers that typically innervate the cornea under normal conditions. This hypothesis develops from our finding that a small proportion of axons regenerating through the nerve grafts reinnervate the cornea.
Despite the high density of the corneal innervation (Rozsa & Beuerman 1982; Guthoff et al. 2005), the cornea is innervated by a relatively small population of sensory neurons that support hundreds of individual nerve endings (de Castro et al. 1998; De Felipe et al. 1999; LaVail et al. 1993; Marfurt et al. 1989; Ivanusic et al. 2013; Launay et al. 2015). The corneal innervation is normally composed of unmyelinated C-fibers and thinly myelinated (Aδ) fibers (Belmonte et al. 2011). Yet, even the Aδ fibers lose their myelination within 1 mm of entering the corneal stroma (Müller et al. 2003). Approximately 66% of the sensory fibers innervating the cornea are unmyelinated C fibers that terminate as polymodal nociceptors (Belmonte 1993; Belmonte & Giraldez 1981; Belmonte et al. 2011). The remaining sensory fibers are cold thermal receptors (10 to 15 %) and mechano-nociceptors (15 to 20 %) (Belmonte et al. 2011). Most corneal nerves contain either Substance P (SP) or calcitonin-gene related peptide (CGRP), but have also been shown to contain pituitary adenylate cyclase-activating peptide (PACAP), galanin, vasoactive intestinal polypeptide (VIP) and met-enkephalin (Müller et al. 2003).

The role of SP on the corneal epithelium has been the most extensively studied of these neuromediators and is known to decrease in patients with corneal hypoesthesia (Yamada et al. 2000; Stone & Kuwayama 1985). Loss of SP from corneal nerve endings also results in changes consistent with NK (Fujita et al. 1984; Shimizu et al. 1987; Donnerer et al. 1996), suggesting that SP helps prevent the breakdown of the corneal epithelium. SP contributes to the maintenance of tight-junctions in the corneal epithelium by upregulating the tight junction proteins E-cadherin (Araki-Sasaki et al. 2000) and ZO-1 (Ko et al. 2009) and may also mediate corneal epithelial adhesion to the basement membrane via integrin upregulation (Nakamura et al. 1998; Chikama et al. 1999). SP promotes corneal epithelial migration after injury via epithelial growth factor receptor (EGFR) activation (Xu & Yu 2011; Yang et al. 2014; Yin et al. 2011) and promotes healing after corneal epithelial injury (Nagano et al. 2003; Nakamura et al. 2003; Nishida et al. 1996). SP and SP analogues in conjunction with insulin growth factor-1 (IGF-1) have been used in several case reports to treat non-healing ulcers in patients, and have demonstrated improved corneal re-epithelization in most studies (Kingsley & Marfurt 1997; Murphy et al. 2001; Morishige et al. 1999; Chikama et al. 1998; Brown et al. 1997; Nakamura et al. 2003; Yamada, Matsuda, Morishige, Yanai, Chikama, Nishida, Ishimitsu & a Kamiya 2008). It is likely that restoration of SP after corneal neurotization is important to promote corneal healing after injury,
and thus it is important to investigate whether nerve fibers reinnervating the cornea after corneal neurotization contain SP.

Nerve growth factor (NGF) may also have an important role in corneal epithelial healing, as it has been shown that topical NGF improves healing of persistent epithelial defects due to impairments of the corneal innervation (Lambiase et al. 1998; Lambiase et al. 2000). The mechanism of how NGF promotes epithelial healing remains unknown and there remains debate regarding the source of NGF as the sensory neurons (Lambiase et al. 1998), corneal epithelium (You et al. 2000), stroma (Lambiase et al. 2000), and the tears have all been reported as sources of NGF in the cornea (Vesaluoma et al. 2000). The neurotrophic factor receptor Trk A has been isolated to the basal epithelium (Touhami et al. 2002), suggesting that NGF may act on basal epithelial cells. Trk A is also known to play a role in the branching of the corneal innervation (de Castro et al. 1998; Albers et al. 1994; Streppel et al. 2002). Neurotrophin-3 (NT-3), and other neurotrophins, have also been isolated to the corneal epithelium (Bennett et al. 1999; Qi et al. 2007), but the significance of this has not been previously investigated.

Corneal neurotization may improve corneal epithelial wound healing by restoring necessary neuromediators such as SP to the cornea or interacting with other corneal cell types to increase the expression of neurotrophins or their associated receptors. The next logical step in investigating corneal neurotization would be to characterize the types of nerve fibers that reinnervate the cornea after surgical corneal neurotization, specifically investigating whether these fibers are myelinated, alter the corneal expression of neurotrophins and/or their receptors, and whether these axons express SP or NGF. We hypothesize that nerves that reinnervate the cornea after corneal neurotization increase corneal expression of SP and NGF.

The first component of this aim will focus on characterizing the nerve fibers that reinnervate the cornea after corneal neurotization. Previous publications investigating the properties of corneal nerve fibers and classifying them as polymodal nociceptors, thermoreceptors or mechanoreceptors, were conducted in cats and utilized dissection of the long ciliary nerves into single afferent fibers (Belmonte & Giraldez 1981; Belmonte 1993). This is not possible in corneal neurotization as it is impossible to dissect single axons from the corneal stroma in order to isolate axons reinnervating the cornea. We have shown that a small number of axons regenerating through the graft reinnervate the cornea. Therefore dissection of single axons from
the graft cannot be used for this purpose. Characterization of nerve fibers will then be limited to the use of immunohistochemistry (IHC) and western blotting (WB) to semi-quantify the amount of protein and RT-PCR to semiquantitate mRNA in the cornea.

We will first identify whether nerves reinnervating the cornea express SP, as this have previously been shown to be the primary neuromediators expressed by the corneal innervation (Belmonete et al. 2011). Preferably, this would be completed as whole mount IHC, but this technique can prove difficult with weaker antibodies in which case we can perform IHC on thin corneal sections. Whole mounts have the advantage of visualizing a larger number of nerve fibers in continuity, whereas thin sections permit the visualization of smaller nerve segments. While this would allow us to determine whether any nerves after corneal reinnervation contain SP and CGRP, it would be difficult to accurately determine in what proportion of nerve fibers they are expressed. Therefore, we can also use WB to quantify the amount of CGRP and SP in the cornea after reinnervation and compare this amount to the uninjured cornea. Thereby, we can obtain an appreciation of to what degree these substances are restored to the cornea after corneal reinnervation.

We can also investigate the corneal expression of neurotrophins (NGF, BDNF, NT-3 and NT-4) and their associated receptors (Trk A, Trk B, Trk C, p75NTR) after corneal neurotization. This has previously been performed using in situ hybridization for p75NTR (You et al. 2000) and immunohistochemistry (Touhami et al. 2002; Qi et al. 2007) in the cornea. Once we identify possible neurotrophins and receptors that are upregulated after corneal neurotization, we can investigate their potential role in corneal healing after corneal neurotization and use IHC to localize their source in the cornea.

**Future direction #2:** Our second future direction is to understand whether corneal reinnervation after corneal neurotization increases the expression of adhesion proteins in the corneal epithelium. In our model of corneal neurotization, differences in healing of the corneal epithelial wound was greatest in the center of the cornea where corneal surface was exposed and not protected by the superior and inferior eyelids. This is consistent with the clinical scenario of NK in which poorly adherent epithelium is characteristic of the disease. Intraepithelial nerves terminate as free nerve endings throughout the layers of the corneal epithelium (Belmonte et al. 2011) and these nerve endings are known to be associated with invaginations of the epithelial
cell membranes, suggesting a possible interaction between the corneal nerves and the corneal epithelium (Müller et al. 2003).

As discussed previously, SP contributes to the maintenance of tight-junctions in the corneal epithelium by upregulating the tight junction proteins, E-cadherin (Araki-Sasaki et al. 2000) and ZO-1 (Ko et al. 2009), and may also mediate corneal epithelial adhesion to the basement membrane through the upregulation of integrins (Nakamura et al. 1998; Chikama et al. 1999). SP, which is expressed by a large number of corneal nerves, also promotes healing after corneal epithelial injury (Nagano et al. 2003; Nakamura et al. 2003; Nishida et al. 1996). Trigeminal ganglion cells influence the expression of collagen VII in in vitro co-culture with rabbit corneal epithelial cells (Baker et al. 1993). The α6β4 integrins are a component of hemidesmosomes in the cornea and the anchoring complex by which the corneal epithelium adheres to the basement membrane. Corneal nerves are known to localize within the β4 integrin-expressing epithelial basal cells (Stepp et al. 2016), and it may be that corneal nerves induce the expression of these proteins in the basal epithelium in a similar manner to how axon-SC interactions influence the upregulation of the α6β4 integrins that facilitate SC adhesion to laminin in the endoneurial tube (Einheber et al. 1993; Salzer 2003)

We hypothesize that corneal neurotization increases the expression of tight junctions (ZO-1 and E-cadherin) in the corneal epithelium as well as the expression of proteins associated with the anchoring complex of the corneal epithelium to the underlying basement membrane (α6β4 integrins and collagen VII). The corneal epithelium in rats that received corneal neurotization will be compared to the epithelium in rats with normal innervation of the cornea and rats in which the cornea is denervated. As with the experiments discussed in Chapters 3 and 4, comparisons will be made four weeks after stereotactic electrocautery of the ophthalmic nerve. Immunohistochemistry will be used to localize the expression of ZO-1, E-cadherin, α6β4 integrins and collagen VII in the cornea. The amount of protein expressed in the cornea can be compared using IHC and WB and the mRNA expression semiquantitated with RT-PCR.

Future direction #3: A third future direction, is to determine the fate of SCs in the donor graft and whether they invade the cornea and contribute to corneal reinnervation. SCs play an important role in peripheral nerve regeneration after injury (Jessen & Mirsky 2016) and in the absence of SC proliferation axon regeneration fails to occur (Hall 1986). Proliferating SCs in the
distal stump form the bands of Bungner that in conjunction with the basal lamina provide a scaffold for regenerating axons to attach and extend (Ide 1996; Fu & Gordon 1997; Navarro et al. 2007). SCs upregulate neurotrophins and glia-derived neurotrophic factor (GDNF) which are important for neuronal survival and axon growth (Chen et al. 2007; Fu & Gordon 1997). During axon growth, the axon develops anchoring connections with the SCs via adhesion molecules including L1, neural cell adhesion molecule (NCAM) and tenascin (Martini et al., 2008) that are necessary for axons to elongate through the endoneurial tubule towards denervated end-organs.

While the role of SCs in peripheral nerve regeneration is well defined within the confines of the endoneurial tubule, the context of peripheral nerve regeneration and corneal reinnervation after corneal neurotization may differ significantly from this paradigm. Axons regenerating into the cornea do not have a distal endoneurial tubule along which to grow, and it remains unknown how axons interact with SCs after corneal reinnervation. It is possible that regenerating axons are attracted to and able to locate non-myelinating SCs already present in the cornea. Alternatively, SCs from the nerve graft may migrate into the cornea and in some way aid reinnervation of the cornea after corneal neurotization. It also remains unknown whether axons after corneal neurotization are myelinated or unmyelinated. We hypothesize that axons reinnervating the cornea after corneal neurotization are unmyelinated and SCs from the nerve graft do not migrate into the denervated cornea to aid in axon regrowth.

Corneal neurotization will be performed in wild-type Sprague Dawley rats using a nerve graft from GFP+ expressing SD rat, permitting the tracing of cells in the donor nerve with fluorescent microscopy as SCs will express green fluorescent protein. Corneal harvest will be performed 4 weeks after V1 electrocautery as described previously. Whole mount immunohistochemistry will be performed with BIII-tubulin to stain corneal axons and S100 for SCs. Co-localization of S100 and GFP will identify donor SCs, and any association with corneal axons can be assessed with the BIII-tubulin axon staining. Additionally, a separate group of animals can be stained with myelin-basic protein (MBP) to identify myelinated axons in the cornea after corneal neurotization. If immunohistochemistry was found to be ineffective, we could investigate the use of flow cytometry to identify S100+ SCs in the normal, denervated and neurotized cornea and determine the number of SCs expressing GFP as a side population.
Future direction #4: A fourth potential direction of this project is to determine whether corneal reinnervation influences corneal epithelial or limbal stem cell proliferation after corneal injury. Similar to the skin, the corneal epithelium is regularly turned over every 7 to 10 days (Hanna et al. 1961; Cenedella & Fleschner 1990). The corneal epithelium is replenished by a stem cell population that continually divides and migrates centripetally to replenish the corneal epithelial cells that are lost to desquamation (Thoft & Friend 1983; Collinson 2004; Di Girolamo et al. 2015). Epidermal growth factor (EGF) signalling is a major pathway mediating corneal epithelial proliferation and migration (Zieske et al. 2000; Nakamura, Nishida, et al. 1997a; Rush et al. 2014; Winkler et al. 2014; Funari et al. 2013; Peterson et al. 2014). Corneal innervation may play a role in EGFR activation after injury (Xu & Yu 2011; Yang et al. 2014).

Limbal stem cells are located within a stem cell niche located within the Palisades of Vogt in the limbus (Ahmad 2012). No consensus exists regarding a standard set of cell markers to characterize limbal stem cells, but the markers ABCD2, p63, p75NTR, TrkA and Hes1 are accepted as distinguishing the limbal stem cell population in vivo (Takács et al. 2009; Schlötzer-Schrehardt & Kruse 2005). ABCB5 was also been found to characterize limbal stem cells (Frank & Frank 2015; Ksander et al. 2014). Limbal stem cells can also be characterized as a side population (SP) by the efflux of Hoeschst 33342 (Ueno et al. 2012). Corneal denervation has also been shown to reduce corneal limbal stem cell number and diminish function (Ueno et al. 2012). It is possible that corneal reinnervation with corneal neurotization increases corneal epithelial proliferation via EGFR or limbal stem cell proliferation.

We hypothesize that corneal reinnervation with corneal neurotization enhances the expression of activated EGFR in the corneal epithelium and increases the number of proliferating cells after wounding of the corneal epithelium. The cornea will be wounded as described in Chapter 4, four weeks after stereotactic electrocautery of the ophthalmic nerve, and the cornea will be harvested 72 hours after wounding. Rats with corneal neurotization will be compared to denervated rats and rats with normal corneal innervation. This tissue has already been harvested from our previous experiments in Chapter 4. IHC and WB will be used to compare phosphorylated EGFR. H&E staining of the entire corneal thickness can be used to measure corneal thickness as a surrogate for corneal proliferation. Cellular proliferation can also be assessed with IHC for Ki67, or alternatively a separate group of animals can be assessed with BrdU injection prior to corneal wounding. Limbal stem cells can be assessed with IHC for limbal stem cell markers, including
ABCG2, ABCB5, p63, K15 and Hes1. Flow cytometry or analysis of stem cell colony-forming efficiency can be performed, although we have not previously used these techniques in our laboratory.

**Future direction #5:** Our final future direction for this project is already underway, and involves the continuation and coordination of clinical research into corneal neurotization between centers using the REDCap system. Due to the rarity of neurotrophic keratopathy, it is necessary to coordinate research efforts with other groups performing corneal neurotization clinically to obtain larger sample sizes of patients and provide greater statistical power to detect differences in ocular surface health. Coordination of research internationally can be performed through REDCap, which is a secure web application for the online building and managing of patient databases.

We expect that other surgeons performing corneal neurotization will also find that corneal reinnervation occurs within three to six months of surgery. Corneal neurotization will improve ocular surface health, as demonstrated by a decrease in the incidence of corneal ulcerations and increase in the success of corneal transplantation in patients with neurotrophic keratopathy. A REB application has been submitted to The Hospital of Sick Children for the construction of an international registry for corneal neurotization. Outreach will be done by the senior authors (Dr. Gregory Borschel and Dr. Asim Ali) to solicit interested surgeons to develop a collaborative research network. A clinic research coordinator will be hired to assist with the maintenance of the online database and ensure patients from other centers are being input into the system properly.

Corneal neurotization is an exciting development in the treatment of NK. Absent innervation in patients with NK, leaves patients susceptible to breakdown of the corneal epithelium, resulting in scarring of the cornea and eventual blindness. By restoring corneal sensation and innervation to the cornea, corneal neurotization may correct the underlying abnormality in these patients by restoring necessary nerve-derived trophic support. The future directions outlined in this thesis will greatly contribute to our understanding of how corneal neurotization influences the corneal epithelium and vision. With further laboratory and clinical investigation, corneal neurotization may become a first-line treatment for patients with NK, and provide these patients with the first treatment option to prevent complications of epithelial breakdown and blindness with NK.
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Appendix

A  Restoration of V2 and V3 trigeminal-distribution cutaneous sensation with cross-face nerve grafts

This chapter is modified from the following:


A.1 Abstract

Trigeminal (CN V) nerve palsy and sensory deficits of the face are an overlooked component of disability. Complete anesthesia leaves patients susceptible to occult injury and facial sensation is an important component of interaction and activities of daily living. Sensory reconstruction is well established in the restoration of hand sensation, however there is only one previous report proposing a surgical strategy for sensory restoration of the face using direct nerve transfers. Nerve transfers, when used alone, have limited application because of their restricted arc of rotation in the face – extending their arc by adding nerve grafts greatly expands their utility. The following cases demonstrate the early results following V2 and V3 reconstruction with cross face nerve grafts (CFNGs) in three patients with acquired trigeminal nerve palsy. All patients demonstrated improved sensation in the reconstructed dermatomes and no patients reported donor site abnormalities. CFNGs result in minimal donor site morbidity and are promising as a surgical strategy to address sensory deficits of the face.
A.2 Introduction

Cross-face nerve grafting (CFNG) is an established surgical treatment for reconstruction of motor function in patients with facial paralysis (Frey & Giovanoli 2002; Fattah et al. 2012). While treating facial motor dysfunction is widely seen as vital to a patient’s quality of life (Lee et al. 2008), little emphasis has been placed on addressing sensory deficits within the trigeminal distribution. Sensory deficits can lead to debilitating parathesia, and complete anesthesia can render the face susceptible to occult environmental injury. Normal sensation is also an important aspect of social interaction and daily activities such as eating and shaving. Sensory abnormalities are difficult to appreciate as, unlike motor or cosmetic concerns, only the patient experiencing them can truly appreciate their impact. Sensory reconstruction is well established to restore hand sensation (Manoli et al. 2014; Paprottka et al. 2013). At the Hospital for Sick Children, Dr. Borschel has operated on three patients to improve sensation to the V2 and V3 sensory distributions with cross-face nerve grafts (CFNGs) using side-to-side coaptations between the affected and unaffected sides. The following cases demonstrate the early results following V2 and V3 reconstruction with CFNGs in three patients with trigeminal nerve palsy.
A.3 Material and Methods

A.3.1 Study Design

We conducted a single-center, retrospective review of patients undergoing maxillary (V2) and mandibular (V3) sensory nerve reconstruction with cross-face nerve grafts at the Hospital for Sick Children / University of Toronto. We measured the efficacy of V2 and V3 reconstruction in a consecutive cohort of patients with either anesthesia or hypoesthesia in the trigeminal nerve distribution of the face. The primary end-point was facial sensation measured with the Semmes Weinstein Enhanced Sensory Test-D (WEST D). We also collected demographic data and data pertaining to etiology of injury.

A.3.2 Clinical Assessments

Patients were evaluated pre- and post-operatively both on the affected and unaffected side of the face using the Semmes Weinstein Enhanced Sensory Test-D (WEST D). Pre-operatively, sensation on both sides of the face was documented in order to identify normal functioning nerves on the contralateral face that could be used as donors. WEST D is an established means of documenting sensory abnormalities in the hand using five filaments that exert progressively greater force. The forces examined are 0.025 g, 0.07 g, 0.5 g, 2 g and 10 g. A force of 0.025 g is generally considered normal sensation for the face, while a force of 0.5 g is considered necessary for protective sensation.

A.3.3 Surgical Procedure

All patients underwent reconstruction of the second (V2) and third (V3) branches of the trigeminal nerve with cross-face nerve grafts from the contralateral infraorbital and mental nerves (Fig. A-1). Bilateral upper and lower buccal sulcus incisions were made and dissection proceeded through a subperiosteal plane to identify the main branches of the infraorbital and mental nerves bilaterally. The infraorbital and mental nerves on the affected side were
transected proximally in order to provide maximal stump length. An epineurial window was made on the medial side of the unaffected mental and infraorbital nerves and approximately one-third of the cross-sectional area of the larger of the two divisions was cut at both sites. The sural nerve graft was then tunneled subcutaneously between the donor and recipient sites and Tisseel fibrin adhesive was used to perform the coaptations in a side-to-end fashion from the donor sensory nerve to the graft, and an end-to-end coaptation, again with fibrin glue, was used to join the distal sural nerve graft to the recipient denervated nerve. All patients also underwent a procedure to re-innervate their insensate cornea on the affected side (Elbaz et al. 2014).

Figure A–1 Cross-Face Nerve Grafts for V2 and V3. The infraorbital and mental nerves (yellow) on the side of sensory loss were transected proximally at their respective foramena. A sural nerve graft (red) was then connected to the unaffected side through a proximal epineurial window in a side-to-end fashion. The free end of the sural nerve graft was then subsequently attached to the transected infraorbital and sural nerves in an end-to-end fashion.
A.4 Results

A.4.1 Case 1

A 16 year-old girl presented with left-sided recurrent corneal ulceration and decreased sensation in the cranial nerve (CN) V distribution following intracranial mengioma resection 4 years earlier. She had a complete motor and sensory CN V palsy. Semmes Weinstein monofilament testing revealed that she had no sensation in the V1, V2 and V3 distributions on the right side of the face and she had normal sensation on the left side of her face. She first reported subjective sensory improvement 6 months following nerve grafting with further sensory recovery at her final follow-up appointment at 2 years. Her facial sensory recovery is illustrated in Figure A-2. There were no complications from the procedure.

Figure A–2 Case 1 Sensory Recovery. Weinstein Enhanced Sensory Test – D (WEST-D) was used to evaluate sensory thresholds. WEST-D contains a set of 5 Semmes Weinstein monofilaments designed to evaluate tactile sensitivity in the face. Testing revealed sensory improvements 2 years post-operatively in almost all distributions with significant sensory improvement to the lower lip.

A.4.2 Case 2

A 16 year-old boy presented to clinic with persistent complete palsies of CN V and incomplete palsies of CN VI and VII following a basal skull fracture 6 years prior. He had persistent corneal
ulcerations refractory to standard treatment and had previous corneal transplants and corrective strabismus surgery. He complained of repeated injury when shaving, and that loss of sensation caused discomfort when eating. Pre-operative testing confirmed absent sensation in the V1, V2 and V3 distributions on the right side of the face. Sensation on the left in the trigeminal distribution was normal. He underwent V2 and V3 reconstruction with cross-face sural nerve grafts from the sides of the contralateral infraorbital and mental nerves into the ends of the recipient denervated infraorbital and mental nerves. Sensation improved in those distributions as demonstrated by the 9-month testing shown in Figure A-3.

**Figure A-3 Case 2 Sensory Recovery.** Objective sensory improvement was apparent 12 months post-operatively with monofilament testing. Sensory improvement was most marked in the upper and lower lip distributions.

### A.4.3 Case 3

A 34 year old woman presented with numbness of the right cheek and upper and lower lips following intracranial tumor resection. She had difficulty chewing on the affected side of her face as she was unable to sense the food bolus and she felt this left her susceptible to inadvertently swallowing or choking on her food. Sensation on the left was normal. She underwent V2 and V3 reconstruction as described in cases 1 and 2. At 4 months, she began to report return of sensation to her upper lip, with objective improvement to the upper lip sensation at 12 months (Fig. A-4). She experienced no operative complications.
**Figure A–4 Case 3 Sensory Recovery.** CN V palsy was partial pre-operatively, and sensory improvements were objectively identifiable starting 12 months post-operatively.
A.5 Discussion

Traumatic injuries (Case 2) and tumors (Cases 1 & 3) are common causes of permanent facial sensory loss (Walker 1990), and may occur as a result of iatrogenic injury (Bagheri et al. 2009; Coulthard et al. 2014; Deppe et al. 2014). To our knowledge, this is the first reported use of CFNGs for sensory reinnervation of the face and sensory reinnervation of V3. Our literature search yielded one case report of trigeminal nerve fascicular transfer for V2 trigeminal nerve sensory deficits, in which Koshima et al. described a technique using fasicular dissection to transfer a distal upper labial branch of the infraorbital nerve and coapting it to a contralateral nasal labial branch to reinnervate a small territory (Koshima et al. 2009). While this technique is effective for the reconstruction of adjacent nerves, more complete reconstruction requires greater nerve length than would be provided by a nerve transfer. CFNGs using the sural nerve permits more proximal reconstruction of the infraorbital and mental nerves allowing potential reinnervation of their entire distribution. Nerve transfers have the advantage of direct neurotization, but they suffer from their restricted arc of rotation, limiting their application (Ray et al. 2010). While CFNG requires donor nerve harvest, there is minimal associated morbidity (Lapid et al. 2007) and the length of harvested nerve grafts permits reconstruction when donor and recipient nerves are not immediately adjacent (Ray et al. 2010; Venail et al. 2009).

An epineurial window and partial axonal injury were used in each case above. While donor nerve injury has the potential to disrupt the normal infraorbital and mental nerves, no donor site sensory abnormalities were reported by any patient, nor were they detected by serial monofilament testing of the donor dermatomal regional. Donor axonal injury may not be required for axon ingrowth into the graft (Akeda et al. 2006; Hayashi et al. 2008; Dvali & Myckatyn 2008); however, increasing nerve injury improves collateral sprouting (Hayashi et al. 2008; Rovak et al. 2001). In these cases, we felt that an epineurial window and axonal injury were necessary to direct axons into the nerve graft to reconstruct large sensory distributions such as these.

Facial sensory deficits may be an overlooked component of disability following multiple cranial nerve injuries. Our patients identified difficulties eating, interacting with others and performing daily activities such as shaving. There were no complication in any of the above treated patients
and the procedure was tolerated well. For those patients with complete CN V palsies, CFNGs for the reconstruction of V2 and V3 deficits has the potential to greatly improve quality of life.
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