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DEVELOPMENT AND VALIDATION OF A RAT MODEL OF CORNEAL REINNERVATION

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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 neurotrophic keratopathy 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/mm^2 ± 12400) in comparison to rats with only ophthalmic nerve electrocautery (2301 µm/mm^2 ± 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.*

Keywords:*Corneal neurotization, ophthalmic nerve electrocautery, contralateral infraorbital nerve*

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INTRODUCTION

Corneal sensation protects the eye from injury and the corneal nerves produce trophic mediators necessary to maintain and repair the corneal epithelium (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). Recurrent and persistent corneal epithelial breakdown in patients with neurotrophic keratopathy 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). neurotrophic keratopathy 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 neurotrophic keratopathy 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). Neuromediatiors 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 neurotrophic keratopathy (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 (Yamada et al. 2008).

Surgical reinnervation of the cornea with donor nerves (i.e. corneal neurotization) restores innervation and sensation to the cornea in patients with neurotrophic keratopathy, thereby addressing the underlying pathophysiology in neurotrophic keratopathy (Samii 1981; Terzis et al. 2009; Elbaz et al. 2014). Reinnervation of the cornea by donor nerves after corneal neurotization in patients with neurotrophic keratopathy has been demonstrated definitively with histology, magnetoencephalography and *in-vivo* confocal microscopy. 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 neurotrophic keratopathy. 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 neurotrophic keratopathy 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 neurotrophic keratopathy 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 neurotrophic keratopathy.

MATERIALS AND METHODS:

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 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.

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)

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 FC™-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 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 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 2 B). The biceps femoris fascia was then repaired using 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 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 3 B). The distal end of the each nerve graft was separately coapted to the right (contralateral) ION (Figure 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 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 B). The overlying conjunctival incisions were then closed to protect the nerve grafts from desiccation and promote revascularization of the grafts (Figure 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.

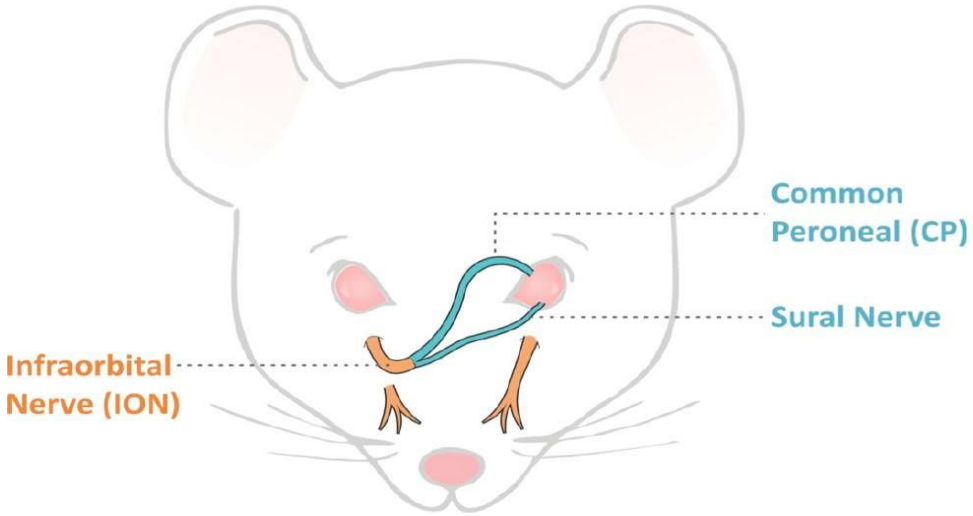

Figure 1 Corneal Neurotization in the Rat.

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.

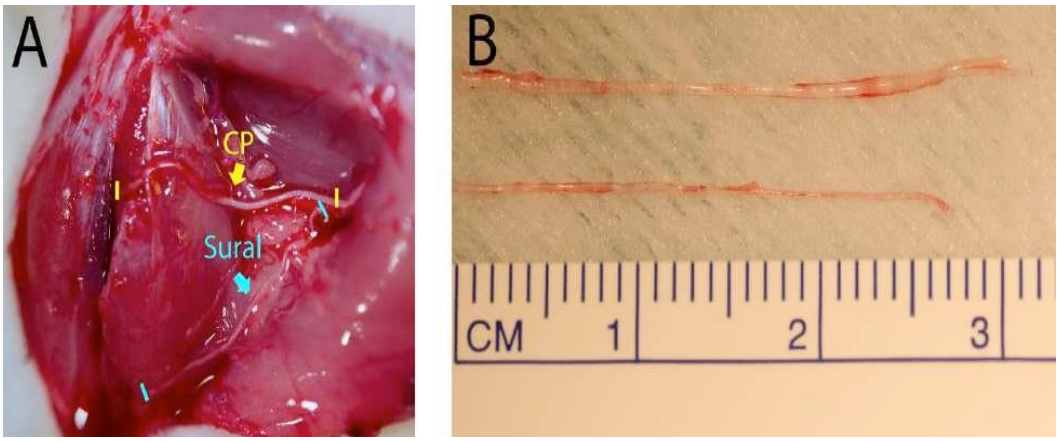

Figure 2 Harvests of the Sural and Common Peroneal Nerve Grafts. Two nerve grafts were harvested from the common peroneal (CP) and sural nerves. The entire length of the CP and sural nerves were exposed by dissecting the biceps femoris away from the quadratus femoris, tibialis anterior and gastrocnemius 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 infraobital nerve coaptation to the left cornea.

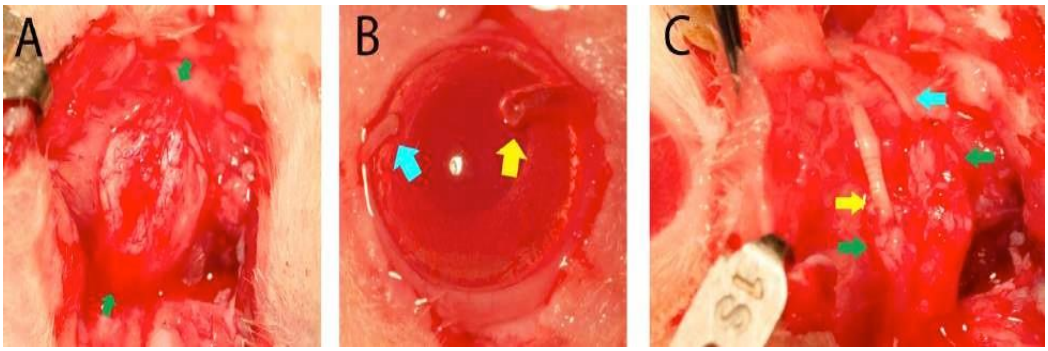

Figure 3 Dissections of the Infraorbital Nerve and Coaptation.

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).

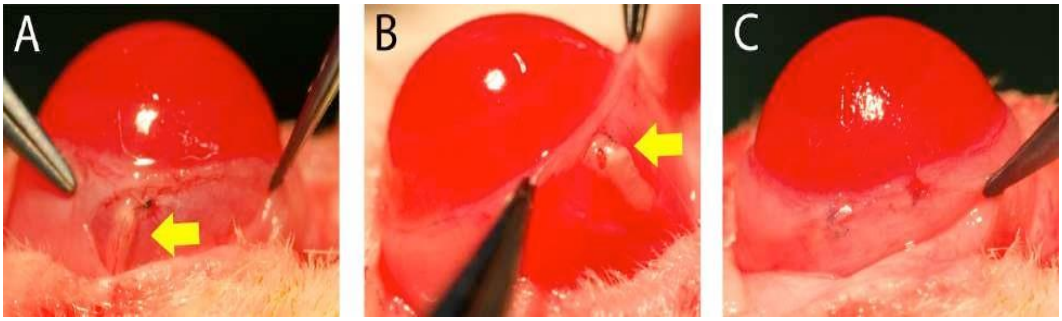

Figure 4 Coaptation of the Nerve Grafts to the Corneal-Scleral Junction.

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. 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 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 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.

Table 1 Determination of Time-point Required for Corneal Reinnervation.

		CN	V1 Electrocautery	Corneal Harvest
Group A	Rat 1	Day 0	Day 28 (+ 4 weeks)	Day 42 (+ 6 weeks)
Rat 2		Day 0	Day 28 (+ 4 weeks)	Day 42 (+ 6 weeks)
Group B	Rat 3	Day 0	Day 28 (+ 4 weeks)	Day 56 (+ 8 weeks)
Rat 4		Day 0	Day 28 (+ 4 weeks)	Day 56 (+ 8 weeks)
Group C	Rat 5	Day 0	Day 42 (+ 6 weeks)	Day 56 (+ 8 weeks)
Rat 6		Day 0	Day 42 (+ 6 weeks)	Day 56 (+ 8 weeks)
Group D	Rat 7	Day 0	Day 42 (+ 6 weeks)	Day 70 (+ 10 weeks)
Rat 8		Day 0	Day 42 (+ 6 weeks)	Day 70 (+ 10 weeks)

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.

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 µm z-stacks (with 10 µm 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.

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 µm using a cryostat (Leica Microsystems Inc., Concord, ON) at -22^0 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).

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).

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 with a surgical microscope, the corneal epithelium was carefully removed with a 0.5 mm burr using the Algerbrush II Fluorescein staining 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.

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 repeated measures ANOVA over time. Statistical significance was accepted at the level of $p < 0.05$; all data are expressed as the mean $\pm$ standard deviation (SD).

RESULTS:

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 5 A/B). Significantly more GFP+ axons were appreciable in the cornea when corneal harvest was performed four weeks after ophthalmic nerve electrocautery (Figure 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.

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 6). This result demonstrates that corneal denervation is necessary for donor axons to reinnervate the cornea.

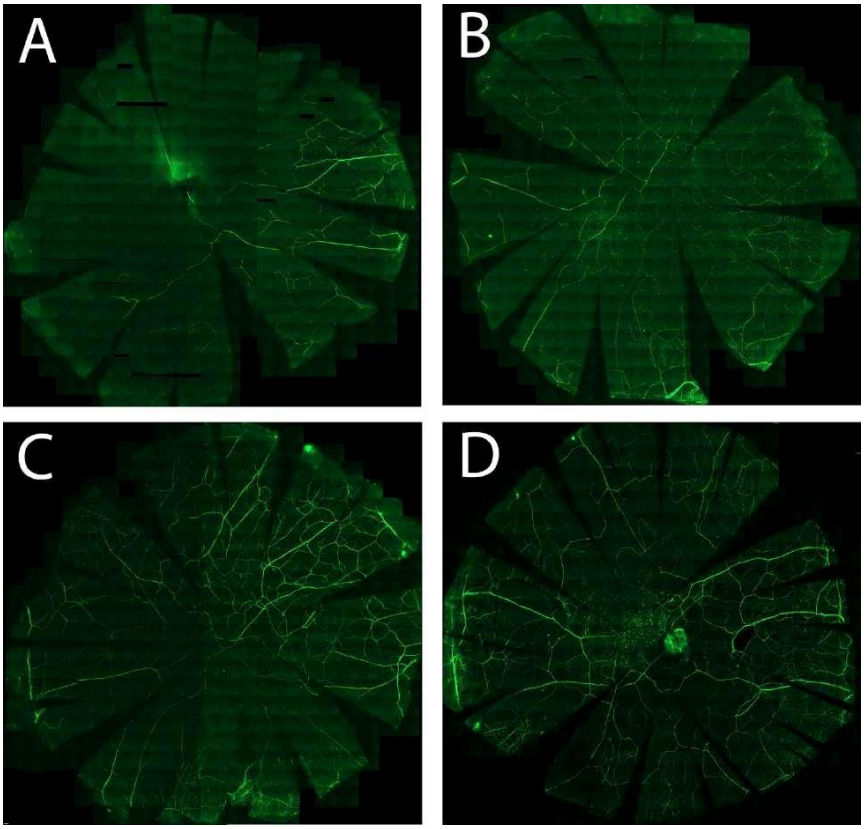

Figure 5 Corneal Reinnervation after Corneal Neurotization.

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.

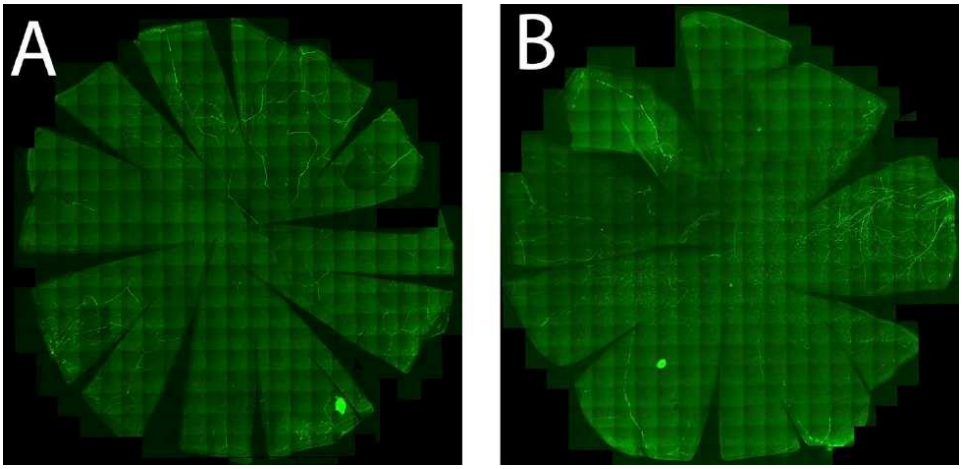

Figure 6 Corneal Reinnervation 10 Weeks after Corneal Neurotization and 1 Week After Ophthalmic Nerve Electrocautery.

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.

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 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 7E-H).

Ophthalmic nerve electrocautery resulted in almost complete loss of sub-basal (101 µm/mm^2 ± 203) and stromal (874 µm/mm^2 ± 990) axons in the center of the cornea in comparison to the sub-basal (26,486 µm/mm^2 ± 3269; $p < 0.0001$) and stromal (20,480 µm/mm^2 ± 5569; $p < 0.0001$) central corneal innervation in uninjured rats. The peripheral sub-basal (285 µm/mm^2 ± 142) and stromal (1,270 µm/mm^2 ± 771) innervation was also significantly reduced in comparison to the peripheral sub-basal (22,458 µm/mm^2 ± 4421; $p < 0.0001$) and stromal (19,470 µm/mm^2 ± 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/mm^2 ± 11987; $p = 0.0068$) and stromal (20,223 µm/mm^2 ± 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/mm^2 ± 1692; $p < 0.0001$) and stromal (26,085 µm/mm^2 ± 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 8.

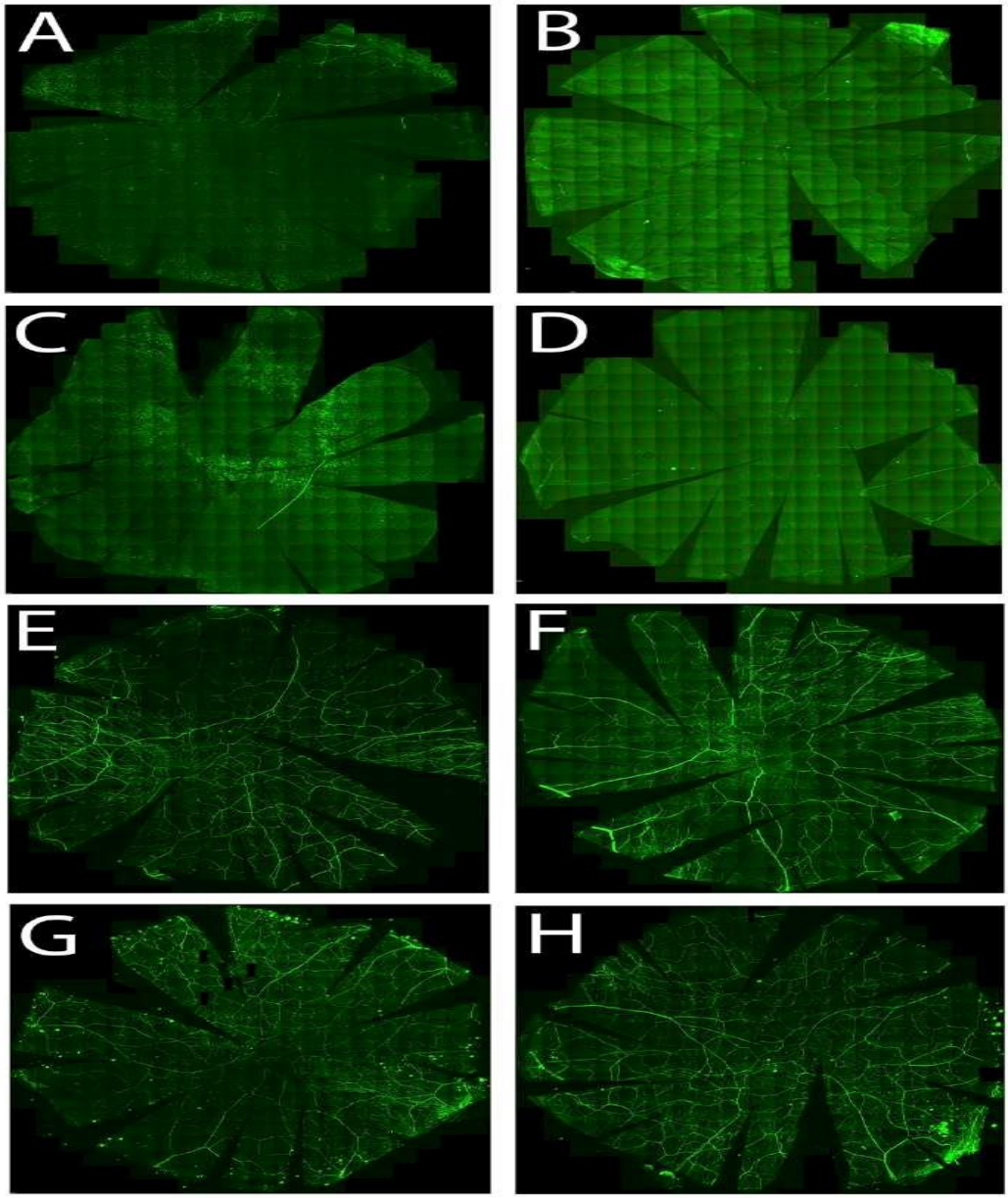

Figure 7 Corneal Reinnervation After Corneal Neurotization in Comparison to Ophthalmic Nerve Electrocautery.

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).

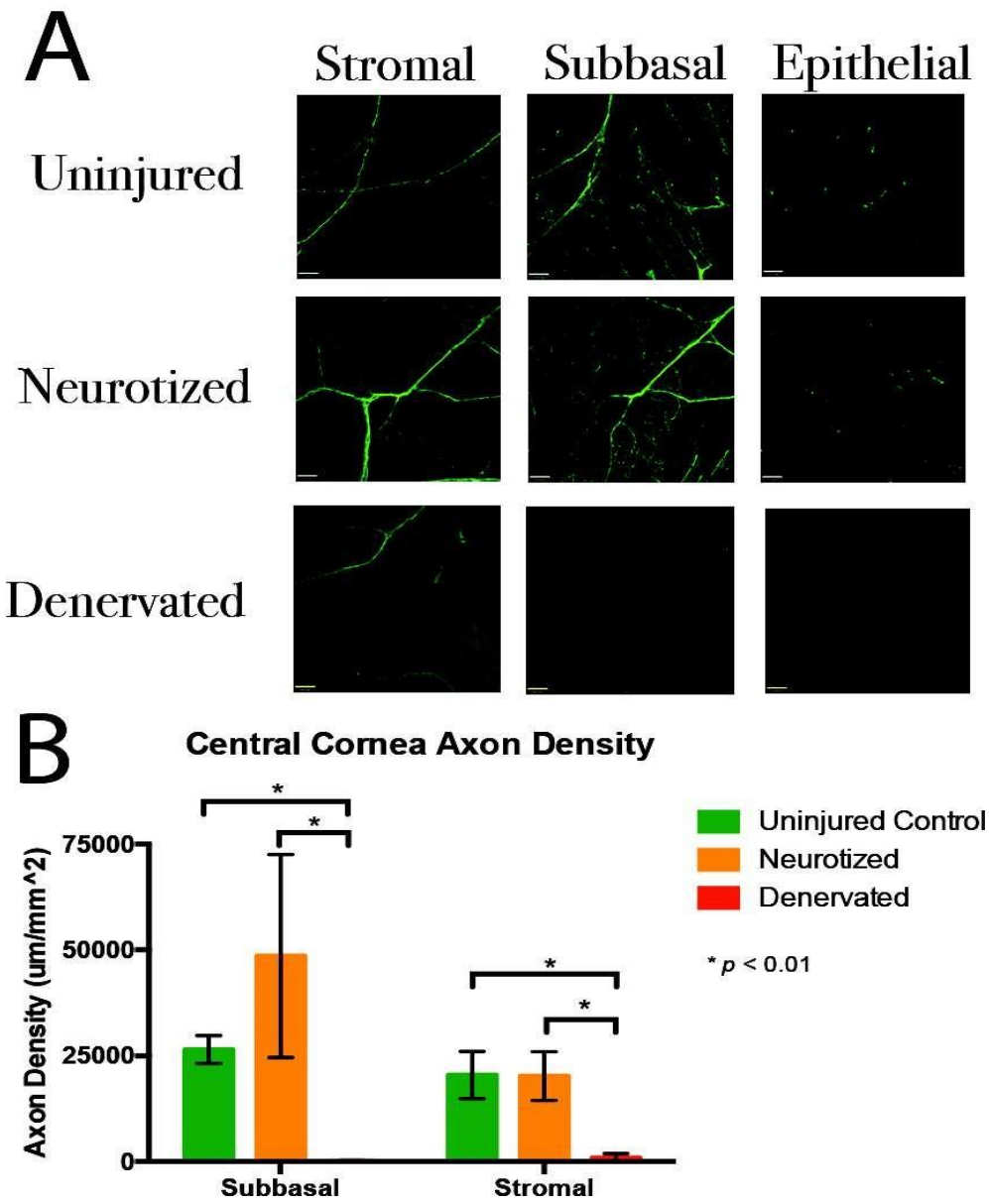

Figure 8 Central Corneal Nerve Density

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)

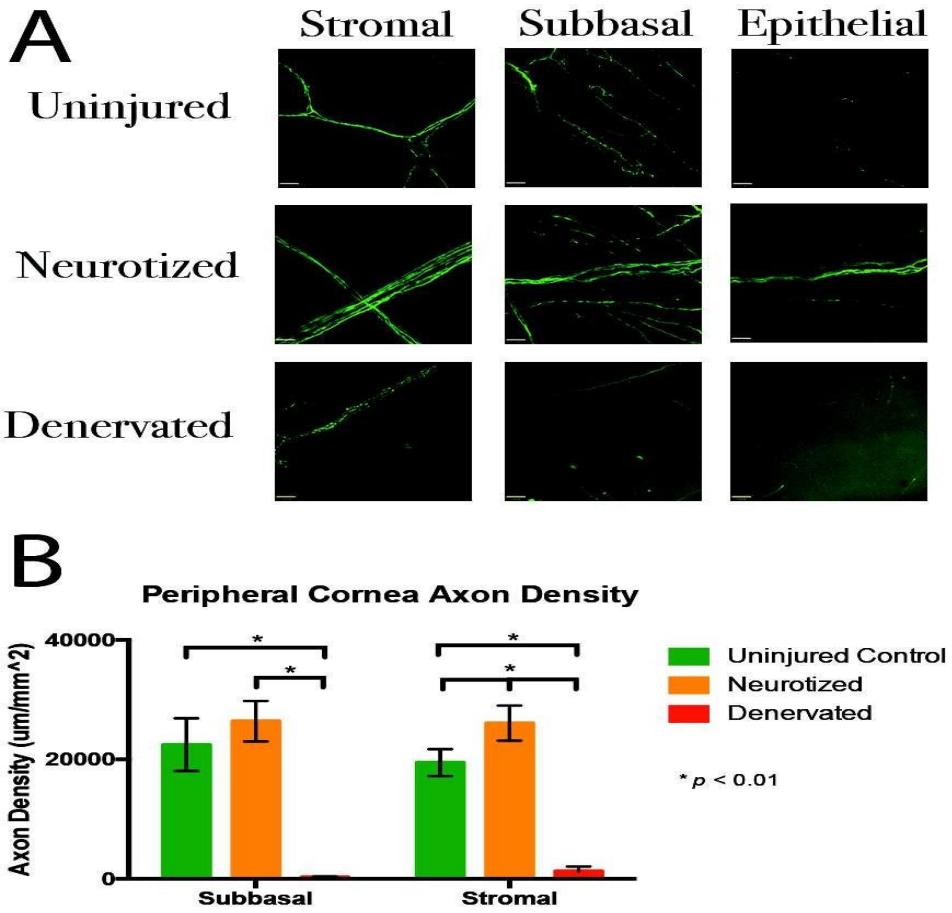

Figure 9 Peripheral Corneal Nerve Density

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 (mm^2) 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)

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

Retrograde labeling data is summarized in Table 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 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 10

Table 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.

	Uninjured	V1 electrocautery	CN
Left TG	219 ± 36	5 ± 3.9	1 ± 1.0
Right TG	0	0	206 ± 82.0

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 3.

Table 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)

	Common Peroneal	Sural
Axon Number	5577 ± 647	2430 ± 613
Axon Diameter	1.37 ± 0.04	1.81 ± 0.14
Fibre Diameter	2.55 ± 0.10	2.39 ± 0.25
Axon Area	1.7268	1.2836
Total Fibre Area	5.5339	4.8771
G-ratio	0.5373	0.4932

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 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 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 11B). Moreover, no rat with corneal neurotization developed a corneal perforation, and two rats (33 %) demonstrated complete wound healing by 96 hours.

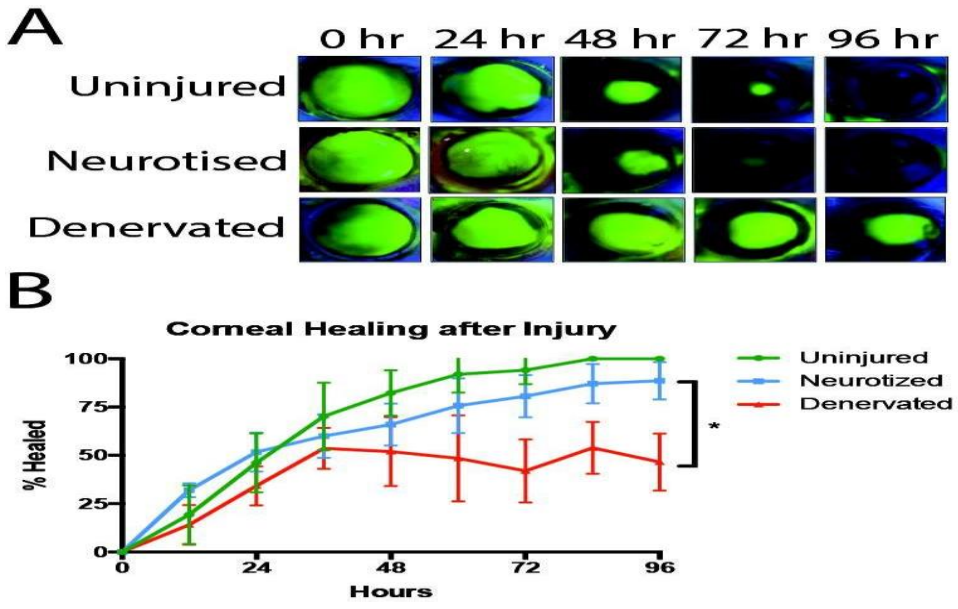

Figure 11 Corneal Healing After Corneal Neurotization.

cornea was completely de-epithelized in i) uninjured rats (with normal corneal innervation), ii) denervated rats (with only 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; * $p < 0.01$).

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 neurotrophic keratopathy 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 neurotrophic keratopathy by restoring innervation to the cornea, thereby restoring neurotrophic support and preventing breakdown of the corneal epithelium, subsequent corneal scarring and vision loss.

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 infraorbital nerve. 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.

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 neurotrophic keratopathy.

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