Corneal Neurotization Improves Ocular Surface Health in a Novel Rat Model of Neurotrophic Keratopathy and Corneal Neurotization

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Corneal neurotization is a novel surgical procedure that reinnervates the cornea in patients with neurotrophic keratopathy (NK). We developed a rat model of NK and corneal neurotization to further investigate corneal neurotization as a treatment to improve maintenance and healing of the corneal epithelium.

METHODS. Thy1-GFP+ Sprague Dawley rats were used to develop the model. Corneal denervation was performed via stereotactic electrocautery of the ophthalmomaxillary branch of the trigeminal nerve. Corneal neurotization was performed by guiding donor sensory axons from the contralateral infraorbital nerve into the cornea via two nerve grafts. Corneal imaging, including nerve density measurements and retrograde labeling were performed to validate the model. In vivo assays of corneal maintenance and repair were used to examine whether treatment with corneal neurotization improved healing in rats with NK.

RESULTS. Corneal neurotization significantly increased corneal axon density in rats with NK (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. Corneal reinnervation after corneal neurotization improved corneal epithelial maintenance and corneal healing after injury (P < 0.01).

CONCLUSIONS. Donor nerve fibers reinnervate the insensate cornea after corneal neurotization and significantly improve corneal maintenance and repair. This model can be used to further investigate how corneal neurotization influences epithelial maintenance and repair in the context of NK.

Keywords: corneal neurotization, neurotrophic keratopathy, corneal nerve

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Neurotrophic keratopathy (NK) is a degenerative corneal disease that develops in patients with impaired or absent corneal innervation. The corneal nerves protect the eye from injury and produce trophic mediators essential for the maintenance and healing of the corneal epithelium.1,2 Patients with NK develop persistent breakdown of the corneal epithelium3–6 and poor healing,6–10 that, in turn, inevitably cause scarring and opacification of the cornea with permanent and irreversible vision loss in many patients.11–15 Conventional ophthalmic management does not address the underlying absence of corneal innervation, and patients often continue to develop progressive vision loss. NK remains one of the most difficult ophthalmic conditions to treat and a leading cause of corneal blindness worldwide.14

Animal models of NK have shown that the corneal epithelium is susceptible to breakdown6,10 and healing is impaired following denervation.6,8–10 Several neuromodulators that are released from sensory axons innervating the cornea have been proposed as regulators of corneal epithelial maintenance and healing.1,2 Some of these, including nerve-derived growth factor and substance P, have been applied to the cornea in patients with NK where they stimulate healing of persistent corneal epithelial ulcerations.18–20 With these treatments, patients remain dependent on daily topical application and they fail to address the absence of protective corneal sensation in the patients with NK.

Corneal neurotization is a novel surgical procedure that addresses the underlying cause of NK by innervating the cornea with donor sensory nerves from elsewhere on the face. Several studies have shown that various techniques of corneal neurotization improve corneal sensation and ocular surface health.21–26 Clinical studies to date are limited by very small sample sizes and the absence of a control group, with patients receiving conventional ophthalmic treatment in addition to corneal neurotization. Hence, although corneal neurotization appears to innervate the cornea and restore corneal sensation, the critical question of whether donor nerves innervating the cornea also contain the necessary
nerve-derived mediators to improve ocular surface health has not been addressed.

Further investigation is necessary to determine whether donor nerves innervating the cornea after corneal neurotization contain the essential neuromodulators to improve corneal epithelial maintenance and ocular surface health. The objectives of this study were to develop the first animal model of NK and corneal neurotization in rats and to use this model to investigate whether corneal neurotization prevents corneal epithelial breakdown and, thereby, improves healing after injury.

METHODS

Animals and Experimental Design

Eighty-six Thy1-GFP+ Sprague Dawley (SD) rats (250–300 g) were used for the development of an animal model of NK and corneal neurotization in the rat. The Thy1-GFP+ rat expresses green fluorescent protein in axons, which permits the visualization of the native corneal innervation, as shown in Supplementary Figure S1, and reinnervation of the cornea with corneal neurotization after corneal denervation. Establishing a model of NK and corneal neurotization included determining the correct stereotactic coordinates and the appropriate settings for electrocautery ablation of the ophthalmomaxillary nerve, as well as the length of time required for corneal reinnervation from the donor nerve. An additional 24 Thy1-GFP+ Sprague Dawley rats (250–300 g) were used for the validation of the NK model, and 42 Sprague Dawley rats (250–300 g) were used to examine corneal epithelial maintenance and healing after injury.

All rats were maintained in a temperature- and humidity-controlled environment with a 12:12 hour light:dark cycle and received ad libitum water and standard rat chow (Purina, Mississauga, ON, Canada). Surgical procedures were conducted in an aseptic manner with an operating microscope (Leitz, Willowsdale, ON, Canada) under inhalational anesthetic (2% isoflurane in 98% oxygen; Halocarbon Laboratories, River Edge, NJ, USA). Rats were provided with buprenorphine (1 mg/kg; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA) for pain relief after all surgical procedures. Rats were euthanized at study termination under deep anesthesia by using intraperitoneal Euthanyl (sodium pentobarbital, 240 mg/mL concentration, 1 mL/kg; Bimeda-MTC, Cambridge, ON, Canada). Experiments were approved by The Hospital for Sick Children Laboratory Animal Services, which adheres to the guidelines of the Canadian Council on Animal Care. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgical Procedures

Stereotactic Electrocautery of the Ophthalmomaxillary Nerve. As previously described in mice and rats, an animal model of NK was developed by ablation of the native corneal innervation (i.e., corneal denervation) by using stereotactic electrocautery of the ophthalmomaxillary nerve in rats. Rats were mounted on a stereotactic frame (Harvard Apparatus, Holliston, MA, USA) and a midline cranial incision was made to identify bregma (intersection point of the coronal and sagittal sutures). A 1-mm burr hole was made at the coordinates anterior-posterior (AP) + 1.5 mm, mediolateral (ML) + 2.0 mm. Coordinates were confirmed with dissection on rat cadavers. An insulated 22-G monopolar electrode (SP 3/50; Pajunk GmbH, Geisingen, 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, Fridley, MN, USA) was used to ablate the ophthalmomaxillary nerve (10 W for 60 seconds). The electrode was then removed and the skin sutured. A complete tarsorrhaphy (suturing together the eyelids) was performed to protect the denervated cornea. Ablation of the corneal innervation was confirmed by an absent blink reflex to touch and cold saline under light anesthesia after confirming an intact reflex on the contralateral eye. The stereotactic electrocautery of the ophthalmomaxillary nerve described above was repeated 3 weeks after the initial procedure to ensure that no regeneration of the native corneal innervation had occurred 4 weeks after the initial stereotactic procedure, when tissue analysis was performed.

Corneal Neurotization. The left sural and common peroneal (CP) nerves were exposed through a mid-lateral thigh incision, and a ~30-mm segment of each nerve was harvested. The right (contralateral) infraorbital nerve (ION) was exposed through an incision parallel to the proximal whisker pad and it was transected distally. The sural and CP nerve autografts were coapted to the transected ION and tunneled subcutaneously toward the left eye and adjacent to the left cornea via a peri-limbal incision (Supplementary Figs. S2A–C). Each nerve graft was sutured to the corneal-scleral junction (Supplementary Fig. S2D). All incisions were closed, permitting revascularization of the nerve graft. All rats received a protective tarsorrhaphy after neurotization. Stereotactic electrocautery of the ophthalmomaxillary nerve was performed 6 weeks after corneal neurotization to provide time for regenerating nerve fibers to grow through the nerve grafts prior to corneal denervation. Stereotactic electrocautery of the ophthalmomaxillary nerve was repeated 3 weeks later (i.e., 9 weeks after corneal neurotization) to ensure that no regeneration of the native corneal innervation had occurred 4 weeks after the initial stereotactic procedure, which is when tissue analysis was performed.

Ophthalmomaxillary Nerve Gross Pathology and Histology

During the surgeries that were carried out to establish the rat model of NK, injury to the ophthalmomaxillary nerve after V1 electrocautery was confirmed with cadaveric dissections and histology. Rats were perfused with normal saline and 4% paraformaldehyde (PFA) and the trigeminal nerve was transected distally. The stereotactic electrocautery of the ophthalmomaxillary nerve was repeated 3 weeks later (i.e., 9 weeks after corneal neurotization) to ensure that no regeneration of the native corneal innervation had occurred 4 weeks after the initial stereotactic procedure, which is when tissue analysis was performed.

Quantification of Corneal Nerve Density

Four weeks after stereotactic electrocautery of the ophthalmomaxillary nerve, whole globes were harvested and immersed 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, Canada). The slides of the corneal whole mount were imaged using a confocal microscope (Olympus IX81, Olympus Life Sciences, Waltham, Massachusetts, USA) with a 10× objective. A minimum of three locations distributed evenly in the peripheral cornea and two locations from the central cornea were imaged with 1-μm Z-stacks of the entire corneal thickness. All images were used for analysis.

Z-stacks were separated into stromal, subbasal, and epithelial layers by using Volocity software (Perkin-Elmer, Waltham, MA, USA). Images were analyzed separately with
ImageJ and NeuronJ plugin to calculate corneal nerve density (in μm/mm²) as described previously. Briefly, images were imported into NeuronJ and the entire length of each GFP-axon was traced to calculate total nerve length and axon density for each image. Additionally, the entire corneal whole mounts were imaged using a confocal microscope (Olympus IX81, Olympus Life Science) 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 together using Velocity software (Perkin-Elmer), and image scales were set to produce an entire image of the corneal reinnervation for analysis of corneal reinnervation after corneal neurotization.

Retrograde Labeling of Neurons Innervating the Cornea

Retrograde labeling of the neurons that reinnervate the cornea was performed 4 weeks after initial stereotactic ablation of the ophthalmomaxillary nerve. The following protocol was modified from previous methods of retrograde-labeling. Filter paper (4 mm in diameter) was soaked in 70% ethanol, positioned on the center of the corneal surface, and left in place for 30 seconds. The disc was then withdrawn and the corneal epithelium carefully removed with a number 15 scalpel blade. Immediately afterward, a piece of absorbable gelatine sponge (Gelfoam; Pfizer Canada, Inc., Kirkland, Canada) that was soaked in 4% FluoroGold (Fluorochrome, LLC, Denver, CO, USA), was placed on the wounded area for 1 hour. The cornea and wound were rinsed three times with sterile saline. Rats were euthanized 7 days after retrograde labeling and perfused with 4% PFA. The ablated left (ipsilateral) and uninjured right (contralateral) trigeminal ganglia (TG) were harvested and postfixed in 4% PFA for one day and cryoprotected in 30% sucrose in 4% PFA for 4 days prior to embedding in optimal cutting temperature compound (Sakura Fine Technical Co., Torrence, CA, USA). The TG were serially sectioned at 20 μm by using a cryostat (Leica Microsystems, Inc., Concord, ON, Canada) at −22°C and mounted onto Superfrost slides (Fisher Scientific). Retrograde-labeled sensory neurons in the TG were counted using an epifluorescence microscope with a 10× objective (100× overall magnification; Leica). A blinded observer performed all counts and a correction was made for double counting by using a previously described correction factor by Abercrombie.

Nerve Graft Histomorphometry

Three nerve samples, 3 mm in length, were harvested from the sural and CP nerve autografts prior to euthanization and fixed in 2.5% glutaraldehyde and buffered in 0.025 M cacodylate overnight, washed, and then stored in 0.15 M cacodylate buffer. Samples were fixed in 2% osmium tetroxide, washed in graded alcohols, and embedded in EPON. Transverse sections at 1-μm thickness were cut through the center of the nerve sample and stained with toluidine blue. Cross-sections were photographed under light microscopy (1000×) by using Image Pro Plus software (MediaCybernetics, Bethesda, MD, USA) and the images analyzed by a blinded observer using MATLAB software (Mathworks, Inc., Natick, MA, USA).

In Vivo Analysis of Corneal Epithelial Breakdown

Sprague Dawley rats with NK were randomized to receive either no treatment (n = 10) or treatment with corneal neurotization (n = 10). Four weeks after stereotactic ablation, the left corneal tarsorrhaphy (eyelid closure) that had kept the corneal surface protected after stereotactic corneal denervation was removed. Fluorescein staining (DioFluor Strips; Innova Medical Ophthalmics, Inc., Toronto, Canada) and digital imaging (Nikon D 5100; Nikon, Tokyo, Japan) were performed daily for 7 days to monitor corneal epithelial breakdown, corneal scarring, and perforation. Imaging was performed with a standardized frame, keeping the camera a fixed distance from the ocular surface. The size of corneal epithelial breakdown was calculated using ImageJ and standardized to the cornea size.

In Vivo Corneal Healing Assay

Rats with and without corneal neurotization were anesthetized 4 weeks after the initial stereotactic electrocautery, and the cornea was assessed for a blink reflex with cold saline and a corneal esthesiometer (Luneau Ophthalmologie, Chartres, France). The corneal epithelium was carefully removed with a 0.5-mm burr by using Algerbrush II (Alger Company, Inc., Lago Vista, TX, USA). Fluorescein staining (DioFluor Strips, Innova Medical Ophthalmics, Inc.) and digital imaging (Nikon D 5100; Nikon) were performed immediately and 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.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 6.0 for Mac (GraphPad Software, Inc., San Diego, CA, USA). All data were analyzed using a 1-way ANOVA with post hoc Bonferroni correction. Wound size was analyzed using 1-way 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).

RESULTS

Stereotactic Electrocautery of the Ophthalmomaxillary Nerve Results in Corneal Denervation and NK

Following stereotactic electrocautery of the left ophthalmomaxillary nerve, gross pathology identified nerve degeneration distal to the site of injury (Fig. 1A). H&E staining of longitudinal sections of the left ophthalmomaxillary nerve demonstrated a cavitating lesion at the injury site and extensive disruption of nerve fiber architecture (Figs. 1B, 1D) in comparison to the right (uninjured) ophthalmomaxillary nerve (Figs. 1C, 1E). In rats in which the cornea was not protected with a complete tarsorrhaphy (eyelid closure), corneal changes consistent with NK developed, including persistent corneal epithelial ulceration, keratitis, neovascularization, and corneal perforation. Comparison of the denervated left cornea with the intact right cornea by using immunofluorescent microscopy 4 weeks after stereotactic electrocautery of the left ophthalmomaxillary nerve demonstrated near complete loss of GFP+ nerve fibers in the denervated cornea (Fig. 2B). The innervation pattern in the uninjured (normal) corneal innervation was highly organized and showed a typical whorl of the subbasal corneal innervation (Fig. 2A).
Donor Nerves Innervate the Cornea After Corneal Neurotization

In Thy1-GFP+ rats, the reinnervation of the cornea 4 weeks after stereotactic electrocautery of the ophthalmomaxillary nerve is obvious in the whole mounts of the cornea (Fig. 2C). Corneal neurotization significantly increased central stromal and subbasal nerve fiber densities following stereotactic ablation of the ophthalmomaxillary nerve in comparison to rats with stereotactic electrocautery of the ophthalmomaxillary nerve alone (P < 0.0001) (Figs. 3A, 3B). The central corneal nerve density in rats with corneal neurotization was not qualitatively different than rats with normal corneal innervation. However, the innervation density was not as uniform, the
axons appeared thinner, and there was loss of the typical whorl pattern of the subbasal nerve plexus. The findings were qualitatively similar for the peripheral cornea (Fig. 3C).

**Donor Sensory Neurons Reinnervate the Cornea After Corneal Neurotization**

Application of dye to the normal (uninjured) left cornea in Thy1-GFP+ rats retrogradely labeled sensory neurons exclusively in the left (ipsilateral) TG; there were no neurons retrogradely labeled in the right (contralateral) TG (Table 1). Retrograde labeling of the left cornea 4 weeks after stereotactic electrocautery of the ophthalmomaxillary 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 were found in the right (contralateral) TG. These findings are consistent with significant corneal denervation after stereotactic electrocautery of ophthalmomaxillary nerve.

In contrast, retrograde labeling of the left cornea 4 weeks after stereotactic ablation of ophthalmomaxillary nerve in rats with left corneal neurotization labeled almost no neurons in the left (ipsilateral) TG and a significant number of neurons in the right (contralateral) TG (Table 1). This finding confirms that nerve fibers from sensory neurons innervating the cornea after corneal neurotization arose from the donor right (contralateral) ION.

**A Small Proportion of Donor Sensory Nerve Fibers Reinnervate the Cornea**

Following the placement of the CP and sural nerve autografts between the proximal stump of the ophthalmomaxillary nerve and the denervated left cornea, myelinated nerve fibers regenerated through the CP nerve graft ($5577 \pm 647$) in comparison with the sural nerve graft ($2430 \pm 613$). The total number of nerve fibers regenerating through the grafts ($\sim 8000$) was significantly higher than the number of neurons found to reinnervate the cornea after corneal neurotization with retrograde labeling ($207 \pm 82$). This suggests that a small proportion of regenerating nerve fibers from the donor ION may reinnervate the cornea after corneal neurotization.

**FIGURE 3.** Imaging of the central cornea demonstrated near complete loss of GFP+ axons in the stroma and complete loss of central subbasal axons after stereotactic electrocautery of the ophthalmomaxillary nerve (i.e., “denervated”) (A). Corneal neurotization (i.e., “neurotized”) rats demonstrated significantly increased density of GFP+ axons in the subbasal and stromal cornea, and this was comparable to the uninjured normal corneal innervation (i.e., “uninjured”) (B). Quantification of axon density as the total nerve fiber length ($\mu$m) per area ($mm^2$) demonstrated that the subbasal and stromal corneal innervation in neurotized rats were significantly higher than denervated animals and comparable to the uninjured normal cornea. *$P < 0.01$. Scale bar: $44 \mu$m.
Corneal Neurotization as a Treatment for NK

**TABLE 1.** Number and Location of Labeled Neurons After Retrograde Labeling of the Left Cornea*

<table>
<thead>
<tr>
<th>Trigeminal Ganglion (TG)</th>
<th>Normal Corneal Innervation</th>
<th>Corneal Denervation†</th>
<th>Corneal Neurotization‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left TG</td>
<td>219 ± 36</td>
<td>5 ± 4</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Right TG</td>
<td>0</td>
<td>0</td>
<td>206 ± 82</td>
</tr>
</tbody>
</table>

* Comparison is made to the cornea in rats with normal, unjured, corneal innervation (i.e., normal corneal innervation). Data are presented as mean ± SD.
† Rats 4 weeks after stereotactic ablation of the ophthalmomaxillary nerve alone (i.e., corneal denervation).
‡ Rats 4 weeks after stereotactic ablation of the ophthalmomaxillary nerve and treatment with corneal neurotization (i.e., corneal neurotization).

**TABLE 2.** Comparison of the Incidence of Corneal Epithelial Breakdown, Corneal Perforation, and Area of Corneal Epithelial Breakdown/Ulceration in Rats

<table>
<thead>
<tr>
<th></th>
<th>NK† (n = 5)</th>
<th>NK + CN‡ (n = 10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence of epithelial breakdown, n (%)</td>
<td>5 (100)</td>
<td>2 (20)</td>
<td>0.007</td>
</tr>
<tr>
<td>Incidence of corneal perforation, n (%)</td>
<td>4 (80)</td>
<td>0 (0)</td>
<td>0.0037</td>
</tr>
<tr>
<td>Area of corneal ulceration, %*</td>
<td>6)</td>
<td>6)</td>
<td>6)</td>
</tr>
<tr>
<td>Day 1</td>
<td>0 ± 0</td>
<td>0.72 ± 2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Day 2</td>
<td>13.8 ± 12.23</td>
<td>0.80 ± 1.976</td>
<td>0.0065</td>
</tr>
<tr>
<td>Day 7</td>
<td>30.07 ± 12.71</td>
<td>0 ± 0</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

* Data are presented as mean ± SD.
† Rats with NK not treated with corneal neurotization.
‡ Rats with NK treated with corneal neurotization.

Corneal Neurotization Prevents Corneal Epithelial Breakdown in Rats With NK

Seven days after removal of the protective tarsorrhaphy and exposure of the left denervated cornea, all rats with NK not treated with corneal neurotization (n = 5) developed breakdown of the corneal epithelium, as assessed with fluorescein staining, and 80% of rats developed a corneal perforation, signifying advanced NK. In comparison, only two rats with NK treated with corneal neurotization (n = 10) developed breakdown of the corneal epithelium (P = 0.007). In both of these two rats in which the denervated left cornea was neurotized, the corneal ulcerations healed by day 7. All the rats with left corneal neurotization demonstrated significantly less corneal epithelial breakdown than rats without treatment at 7 days after corneal exposure (0.0 ± 0.0 vs. 30.1 ± 12.7, P < 0.0001). Furthermore, none of the rats in which the left cornea was treated with corneal neurotization developed corneal perforations in comparison with 80% of the rats in which the denervated cornea was not treated (P = 0.003). Corneal neurotization of denervated cornea also significantly decreased corneal scarring. Data are summarized in Table 1. Figure 4 contains representative images demonstrating significant corneal ulceration (Fig. 4A) and scarring (Fig. 4B) in the left cornea of a rat with denervation of the left cornea. Figure 4A and 4B also show representative images demonstrating decreased corneal ulceration (Fig. 4A) and scarring (Fig. 4B) in the left cornea of a rat treated with corneal neurotization. Treatment with corneal neurotization significantly decreased corneal ulceration (Fig. 4C).

Corneal Neurotization Improves Healing of the Corneal Epithelium in Rats With NK

Following corneal de-epithelization in rats with NK, the corneal epithelium healed more quickly in rats in which the cornea was treated with corneal neurotization than in rats that were not treated with corneal neurotization. Representative images of the corneal wound in each group are shown in Figure 5A. In rats with normal (i.e., uninjured) corneal innervation, the corneal wound healed within 96 hours (mean of 72 hours ± 9.2) in all the rats (n = 6) (Fig. 5B). Impaired wound healing was evident in rats after stereotactic ablation of the ophthalmomaxillary nerve with no corneal neurotization treatment; the corneal wound failed to heal in all rats without treatment, with all rats (n = 6) demonstrating corneal perforation prior to the 96-hour time point. The latter is a severe complication of NK. In contrast, the corneal wound in rats with stereotactic ablation of the ophthalmomaxillary nerve that was treated with corneal neurotization (n = 10) healed significantly more quickly than rats in which the denervated cornea was not neurotized; a greater percentage of the wound was re-epithelialized by 96 hours (88% ± 9.7 vs. 47% ± 14.8; P < 0.01) (Fig. 5B). Moreover, corneal perforation was never observed in the rats in which the denervated cornea was neurotized, and two rats (20%) demonstrated complete wound healing by 96 hours.

Discussion

This paper describes the first animal model of corneal neurotization developed in the rat. We developed our model using the Thy1-GFP+ rat because they express green fluorescent protein in axons and have been previously validated for the study of nerve fiber regeneration after injury.27,34 Using this model, we demonstrated that donor nerves reinnervate and restore axon density after corneal neurotization treatment, preventing corneal epithelial breakdown and perforation, and accelerating healing of corneal ulcerations in rats with NK. This novel animal model enables investigation of NK and its potential surgical and nonsurgical treatments.

A rat model of corneal neurotization first necessitated a reliable model of NK that resulted in denervation of the cornea and subsequent keratopathy. We experimented with several models of NK in the rat10,27-29,35 and found that stereotactic electrocautery of the ophthalmomaxillary nerve was the most reliable technique to denervate the cornea while leaving the vascular supply to the ocular surface intact.10,28,35 Stereotactic electrocautery of the ophthalmomaxillary nerve resulted in complete corneal denervation 1 week after injury, but a small amount of corneal reinnervation was apparent after 4 weeks. The source of reinnervation appeared to derive from the ipsilateral ophthalmomaxillary nerve, as a second stereotactic procedure 3 weeks after the first resulted in complete corneal denervation for a period of 4 weeks. Corneal reinnervation was not further investigated after this time, as we required 4 weeks of corneal denervation to permit corneal reinnervation by the contralateral ION, which was the donor nerve used in rats treated with corneal neurotization. Stereotactic ablation of the TG was also attempted to ablate the primary sensory neurons. Although electrocautery of the ophthalmomaxillary nerve was well tolerated, electrocautery of the more proximal TG resulted in unacceptable high rates of morbidity and poor survival, likely due to the proximity to the brainstem.

Rats with corneal neurotization treatment demonstrated corneal nerve density comparable with the uninjured cornea 4 weeks after stereotactic ablation of the ophthalmomaxillary...
nerve. However, qualitatively, the corneal nerve pattern was not as homogeneous or organized as the uninjured cornea, and the subbasal nerve plexus lacked the typical whorl pattern. The organization of subbasal nerve plexus may continue to remodel with time; however, the significance of this difference requires further investigation. Several techniques of corneal neurotization have been described. Neurotization was performed in our model by using the contralateral ION via two autografts, similar to the technique described by Elbaz et al. The contralateral ION was used, as it is robust in the rat, and a contralateral donor permitted the confirmation with retrograde labeling that reinnervation of the cornea was derived from the contralateral donor nerve. Because each graft measured approximately 30 mm in length, the surgical procedure to place the grafts was performed prior to stereotactic ablation of the ophthalmomaxillary nerve. This provided an opportunity for nerve fibers, which regenerate at 1 mm/day, to grow toward the cornea prior to corneal denervation. The number of neurons labeled with retrograde labeling in the uninjured cornea was consistent with other published studies, identifying between 50 and 450 neurons innervating the corneal epithelium. Retrograde labeling of the trigeminal neurons reinnervating the cornea after corneal neurotization confirmed that reinnervation was derived from the donor contralateral ION. Interestingly, the number of TG labeled with retrograde labeling after corneal neurotization (207 ± 82) was significantly less than the mean number of myelinated axons regenerating through the sural and CP nerve graft (8007 ± 1260) identified with histomorphometry, suggesting that a small proportion of axons regenerating through the nerve grafts reinnervate the cornea after corneal neurotization. This hypothesis requires further investigation;
Corneal Neurotization as a Treatment for NK

**A**  
0 hr 24 hr 48 hr 72 hr 96 hr  
Uninjured  
Neurotized  
Denervated

**B**  
Corneal Healing after Injury

Figure 5. The cornea was completely de-epithelized in rats with normal (uninjured) corneal innervation, corneal denervation (with only stereotactic electrocautery of the ophthalmomaxillary nerve), and corneal neurotization (with corneal neurotization and ophthalmomaxillary nerve ablation). Corneal healing was examined 4 weeks after ablation of the ophthalmomaxillary nerve. The de-epithelialized corneal stroma was stained with fluorescein (green) to assess wound size and healing. Healing via corneal re-epithelialization reduces the amount of fluorescein-staining of the underlying stroma. (A) Corneal wound healing occurred more quickly in rats with corneal neurotization than rats with ophthalmomaxillary nerve ablation alone. (B) When wound size was compared over time, corneal healing was significantly improved in rats with corneal neurotization in comparison with denervated rats. *P < 0.01.

However, it is possible that the cornea selectively permits the growth of only unmyelinated nerve fibers with a particular phenotype. The corneal innervation is composed of a highly regulated network of unmyelinated C fibers and a small number of thinly myelinated (Ad) fibers that terminate as free-nerve endings in the corneal epithelium. Unlike the corneal innervation, the donor nerves used to innervate the cornea with corneal neurotization contain a more diverse population of nerve fibers, including a large number of myelinated fibers. To maintain corneal clarity, the growth of myelinated axons into the cornea may be restricted. It is also possible that the receptors in the cornea that guide axon regeneration following corneal neurotization are saturated, restricting further axon growth once a certain number of axons have successfully regenerated into the corneal periphery. Our model can be used to further investigate whether myelinated fibers innervate the cornea after corneal neurotization or whether the cornea selectively regulates innervation after corneal neurotization.

Importantly, we demonstrated in our model that donor nerve fibers innervating the cornea after corneal neurotization decrease breakdown of the corneal epithelium, prevent corneal perforation, and accelerate healing after injury. It is likely that donor nerve fibers restore trophic support to the corneal epithelium normally supplied by the native corneal innervation; although, this explanation is not exclusive and the molecular mechanisms requires further investigation. Potential avenues of investigation include several neuromediators previously proposed as necessary for maintenance and healing of the corneal epithelium, including substance P, calctonin gene-related peptide, and nerve growth factor. Interactions between the corneal epithelium and corneal innervation may also upregulate the expression of 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. Our finding that corneal neurotization improves healing of corneal epithelial injuries in rats with NK is consistent with previous work demonstrating that innervation is necessary for the proliferation of corneal epithelial or limbal stem cells after injury. In our study, we did not observe a return of blink reflex in rats that were treated with corneal neurotization, which may be expected, as the donor ION does not contain necessary synapses to drive reflexive blinking. The impact of corneal neurotization on lacrimation was not investigated in this study and remains unknown; however, our rat model can be used to investigate the impact of corneal neurotization on lacrimation.

Despite developments in the treatment of NK, it remains a major cause of corneal blindness worldwide. Conventional ophthalmic treatment, including the use of topical neuromediators, fails to address the underlying loss of corneal sensation and innervation. Our results provide further evidence that nerve fibers innervate the corneal epithelium after corneal neurotization and improve ocular surface health. These results complement the clinical studies performed in patients undergoing corneal neurotization, and our animal model provides a means of further investigating corneal neurotization to elucidate by what mechanism corneal neurotization improves corneal epithelial integrity and healing after injury. These findings will greatly contribute to our understanding of corneal neurotization and support the use of corneal neurotization as a treatment for NK. Surgical reinervation 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 the debilitating complications of NK.

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**References**


