Patients with facial palsy suffer from functional and aesthetic deficits, including impaired speech, difficulty eating, impaired ability to protect the cornea, diminished social interaction, and reduced quality of life. Facial reanimation surgery is performed in severe cases. Cross-face nerve grafting, where a sural nerve graft is used to conduct axons of a facial nerve branch from the healthy side of the face to the paralyzed side of the face to innervate the mimetic muscles or a free muscle transplant, is the criterion standard for emotional facial reanimation. The sural nerve grafts can be over 20 cm long; therefore, it takes several months for axons to regenerate through them. Histomorphometric studies showed that only 10 to 50 percent of donor axons reach the distal end of the cross-face nerve graft. With only 100 to 200 motor axons reaching the target muscle, the extent of facial movement may be insufficient.

**Background:** In unilateral facial palsy, cross-face nerve grafts are used for emotional facial reanimation. Facial nerve regeneration through the grafts takes several months, and the functional results are sometimes inadequate. Chronic denervation of the cross-face nerve graft results in incomplete nerve regeneration. The authors hypothesize that donor axons from regional sensory nerves will enhance facial motoneuron regeneration, improve axon regeneration, and improve the amplitude of facial muscle movement.

**Methods:** In the rat model, a 30-mm nerve graft (right common peroneal nerve) was used as a cross-face nerve graft. The graft was coapted to the proximal stump of the transected right buccal branch of the facial nerve and the distal stumps of the transected left buccal and marginal mandibular branches. In one group, sensory occipital nerves were coapted end-to-side to the cross-face nerve graft. Regeneration of green fluorescent protein-positive axons was imaged in vivo in transgenic Thy1-green fluorescent protein rats, in which all neurons express green fluorescence. After 16 weeks, retrograde labeling of regenerated neurons and histomorphometric analysis of myelinated axons was performed. Functional outcomes were assessed with video analysis of whisker motion.

**Results:** “Pathway protection” with sensory axons significantly enhanced motoneuron regeneration, as assessed by retrograde labeling, in vivo fluorescence imaging, and histomorphometry, and significantly improved whisker motion during video analysis.

**Conclusion:** Sensory pathway protection of cross-face nerve grafts counteracts chronic denervation in nerve grafts and improves regeneration and functional outcomes. (Plast. Reconstr. Surg. 135: 460, 2015.)
In a large series of free muscle transplantations innervated by cross-face nerve grafts, secondary operations were required in up to 72 percent of patients because of inadequate muscle movement (excursion). Improved muscle excursion, therefore, would be of great benefit to patients undergoing cross-face nerve grafting.

Cross-face nerve grafts undergo chronic denervation because of the long distance and duration of axon regeneration through the grafts. Chronic denervation and chronic axotomy reduce the capacity for nerve regeneration. “Protection” of denervated distal nerve stumps can provide additional donor axons for endogenous support of the denervated nerve. Sensory protection of denervated muscle improved regeneration after surgical repair. However, these authors transected a motor nerve and coapted a sensory nerve to the motor nerve; then, in a second stage, they repaired the original motor nerve. Such strategies are cumbersome and may not be widely accepted or feasible in clinical practice.

We hypothesize that donor axons from regional sensory nerves can “protect” cross-face nerve grafts by counteracting the effects of chronic denervation of the graft and enhance the regeneration through the graft. We aim to improve the histologic and functional outcomes after cross-face nerve grafting in a rat model. The protection of cross-face nerve grafts would provide the neurophysiologic basis for enhancing outcomes of facial reanimation.

**MATERIALS AND METHODS**

**Experimental Animals**

Wild-type Sprague-Dawley rats (Harlan, Indianapolis, Ind.) were used for the histologic and behavioral analysis. *Thy1–green fluorescent protein* rats, raised on a Sprague-Dawley background, were used for in vivo imaging of axon regeneration. These transgenic rats express green fluorescent protein in neurons but not in nonneural cells. All rats weighed 250 to 300 g. The rats (Fig. 1) were divided into three groups: a cross-face nerve graft group, a cross-face nerve graft group with end-to-side coaptation of occipital nerves to the graft, and a sham group (control). Wild-type (*n* = 10 per group) and transgenic rats (*n* = 12 per group) were used in every group. Experiments were approved by the animal care committee of The Hospital for Sick Children, Toronto, Ontario, Canada.

**Animal Surgery**

All operative procedures were performed under 2.5% isoflurane gas anesthesia using aseptic technique. The right common peroneal nerve was harvested as a 30-mm nerve graft through a gluteal muscle-splitting incision. In the sham groups, the common peroneal nerve was exposed but not harvested, and the muscle and the skin were closed with interrupted 5-0 Vicryl (Ethicon, Inc., Somerville, N.J.) sutures. The facial nerves were exposed through a coronal incision, which was extended caudally on the left side of the face to expose the main trunk of the facial nerve. On the right side,

![Fig. 1. Experimental groups. (Left) In the cross-face nerve graft (CFNG) group, a 30-mm cross-face nerve graft bridged the gap between the donor right buccal branch (B) of the facial nerve and the contralateral recipient mandibular (M) and buccal (B) branches of the contralateral facial nerve. (Right) In the protected group, a cross-face nerve graft was treated with two end-to-side sensory occipital (ON) nerves along the graft.](image)
the buccal branch of the facial nerve was dissected for preparation as the donor nerve for the cross-face nerve graft (Fig. 1). All nerve coaptations were performed with interrupted epineural 10-0 nylon sutures. On the recipient side (left), the cross-face nerve graft was coapted end-to-end to the buccal and marginal mandibular branches of the facial nerve (Fig. 1, left). In cross-face nerve graft with occipital nerve coaptation group rats, the left and right occipital nerves were cut and coapted end-to-side to the cross-face nerve graft through an epineural window (Fig. 1, right). The first end-to-side coaptation was performed 10 mm distal to the proximal suture site of the graft and the second one 20 mm distal to it. In the sham group, the facial nerves were exposed but left uninjured. After irrigation, the skin was closed with 5-0 Vicryl sutures.

In Vivo Imaging of Axon Regeneration
The green fluorescent protein–positive axons in transgenic Thy1–green fluorescent protein rats were imaged at the time of cross-face nerve grafting/sham operation and subsequently every 4 weeks postoperatively for 16 weeks. The axons were imaged macroscopically using a green fluorescent protein–MDS-96/BN excitation stand (BLS Ltd, Budapest, Hungary), a camera stand with eight ultraviolet lights. The imaging was performed under 2.5% isoflurane gas anesthesia and meloxicam. The facial nerves and cross-face nerve grafts were reexposed and images captured under natural light and ultraviolet light.

Video Analysis of Whisker Movement
The video analysis of whisker movements was performed as described previously. Videos were recorded preoperatively and 16 weeks postoperatively. Because both the buccal and the marginal mandibular nerve branches supply the whisker pad and the right buccal branch was used as donor nerve for the cross-face nerve graft, the whisker movements of the experimental groups were compared with each other but not with the normal control animals. Videos of whisker movements were recorded for 3 to 5 minutes (60 frames/second) for each rat. Using a two-dimensional video analysis system (PEAK Motus 2000; PEAK Performance Technologies, Inc., Englewood, Colo.), points were tracked during maximal whisker movement (Fig. 2). We analyzed frequency of whisking (protraction and retraction cycles per second), amplitude of whisking (in degrees), angular velocity (in degrees per second) and angular acceleration (in degrees per second).

Retrograde Labeling of Regenerated Neurons
Sixteen weeks after the primary procedure of the insertion of the cross-face nerve graft with and
without the end-to-side coaptation of the occipital nerves (Fig. 1), the left facial nerve was reexposed under isoflurane anesthesia. The buccal and marginal mandibular branches of the left facial nerve were exposed and transected 10 mm distal to the cross-face nerve graft. The nerve stump was placed in a petroleum jelly well containing 4% Fluoro-Gold (Sigma-Aldrich, St. Louis, Mo.) for the buccal branch, or 4% Fluoro-Ruby (Sigma-Aldrich) for the marginal mandibular branch, for 1 hour. After 1 week, the rats were killed and perfused with 0.9% saline and 4% paraformaldehyde. The brainstem and the dorsal root ganglia of the C1 and C2 dorsal roots in the cross-face nerve graft with occipital nerve coaptation group were harvested; the brainstem was cut into 50-μm sections and the dorsal root ganglia was cut into 20-μm sections using a cryostat (Leica, Wetzlar, Germany). The labeled neuron cell bodies in the right facial nucleus were counted in every third section under the fluorescent microscope (20× magnification; Leica) as described previously.29,34 There were no labeled neurons in the left facial nucleus in the control group, which shows that there was no spontaneous regeneration of the left facial nerve. The labeled dorsal root ganglia neurons were counted in every fifth section, and the counts were then corrected as described previously.35 The selection of sensory, cutaneous branches of the occipital nerves (which were selected with intraoperative electrostimulation) was confirmed, as only neurons in the C1 and C2 dorsal root ganglia were labeled retrogradely. There were no labeled motoneurons in the ventral horn of the corresponding segments.

**Histomorphometry of Myelinated Axons**

For histomorphometric analysis, a segment of the left facial nerve was harvested 10 mm distal to the cross-face nerve graft at the time of retrograde labeling (Fig. 3). The nerve samples were fixed in 2% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated with ethanol, and embedded in Araldite 502 (Polyscience, Inc., Warrington, Pa.). After sectioning (0.6 μm) with a LKB II Ultramicrotome (LKB-Produckter A.B., Broma, Sweden), the samples were stained with 1% toluidine blue. At 1000× overall magnification under the light microscope, the entire nerve cross-section was captured and evaluated with image analysis software (Image-Pro Analyzer version 7.0; Media Cybernetics, Bethesda, Md.), as described previously.35,36 The number of myelinated axons, fiber size and distribution, and myelination thickness were analyzed.

**Statistical Analysis**

Descriptive statistics were performed on all metrics; means and standard deviations are reported. Mann-Whitney U (Wilcoxon rank sum)
tests and unpaired t tests were used to compare the results for statistical significance (p < 0.05).

RESULTS

In Vivo Imaging of Axon Regeneration through Cross-Face Nerve Grafts

Early in vivo imaging of regenerating axons in the Thy1–green fluorescent protein rats showed the greatest differences between the cross-face nerve graft group and the group with end-to-side coaptation of the occipital nerves to the cross-face nerve graft during the early time points of nerve regeneration. At 8 weeks (Fig. 4), regenerating axons in the cross-face nerve graft group had not crossed the distal suture site of the 30-mm graft on the left of the rat (Fig. 4, above). In the cross-face nerve graft with occipital nerve coaptation group (Fig. 4, below), axons reached the recipient (left) side of the face across the distal suture site of the cross-face nerve graft. By 16 weeks, green fluorescent protein–positive axons had reached the recipient side in both experimental groups (Fig. 5).

Video Analysis of Whisker Movement during Maximal Whisker Motion

Videos of maximal whisker movements were recorded for both experimental groups preoperatively and after 16 weeks of regeneration (points

Fig. 4. In vivo imaging of regeneration of green fluorescent protein–positive axons after 8 weeks of regeneration. The regenerating axons had not crossed the distal suture site of the unprotected cross-face nerve graft (arrowhead, above), whereas they reached the recipient side in the group with end-to-side coaptation of the occipital nerves (arrows, below) to the graft. (Nerve grafts were imaged on a dark fabric to block the strong fluorescence of the brain through the skull.)
tracked are shown in Fig. 2). At 16 weeks postoperatively, two-dimensional analysis of predefined points (Fig. 2) showed significantly higher amplitudes (Table 1 and Fig. 6, right) and higher velocity during retraction (Table 1) in the cross-face nerve graft with occipital nerve coaptation group. The end-to-side coaptation of sensory nerves to the cross-face nerve grafts did not significantly influence the other evaluated parameters of whisker movement, as described in Table 1, because areas of motion pattern control in the brainstem were not altered.

### Retrograde Labeling of Neurons That Regenerated Their Axons

The retrograde labeling of motoneurons in the facial nucleus after 16 weeks of nerve regeneration failed in two animals in each experimental group (n = 10 per group), because of technical problems, and were excluded from analysis. In the cross-face nerve graft with occipital nerve coaptation group, the dorsal root ganglia of all 10 rats (C1 and C2 bilateral) were analyzed.

The mean number of retrograde labeled motoneurons in the right facial nucleus (Fig. 7, left) was 304.8 ± 23.8 in the cross-face nerve graft group and 402.1 ± 27.6 (p = 0.0184) in the cross-face nerve graft with occipital nerve coaptation group (Fig. 7, above, right). To confirm the ingrowth of sensory nerves into the cross-face nerve graft in the cross-face nerve graft with occipital nerve coaptation group, the C1 and C2 dorsal root ganglia were harvested and the labeled sensory neurons counted in the left and right dorsal root ganglia. The distribution of labeled sensory
The number of labeled dorsal root ganglia neurons was as follows: dorsal root ganglia C1 right, 286.4 ± 196.0 (n = 10); dorsal root ganglia C1 left, 502.1 ± 180.8 (n = 10); dorsal root ganglia C2 right, 158.0 (n = 1); and dorsal root ganglia C2 left, 23.4 ± 22.6 (n = 5). Sensory neurons in all C1 dorsal root ganglia labeled, but only six C2 dorsal root ganglia labeled, indicating constant contribution from C1 but variable contribution of C2 to the occipital nerves (Fig. 7, below, right).

### Histomorphometry of Myelinated Axons

In all groups, the left buccal and marginal mandibular nerves were harvested 10 mm distal to the cross-face nerve graft (except for the control group, in which a segment 10 mm distal to the branching was taken) for histomorphometric analysis after 16 weeks of regeneration (or sham surgery in the control group). The nerve cross-sections from rats in the cross-face nerve graft with occipital nerve coaptation group regenerated significantly greater numbers of myelinated
The mean number of myelinated axons was 1873 ± 107 in the cross-face nerve graft group and 2458 ± 196 (p = 0.0192) in the cross-face nerve graft with occipital nerve coaptation group. There were no significant differences in the fiber and axon diameters, myelin thicknesses, or distributions of fiber diameters between the two groups (Fig. 3). The dotted line in Figure 3 indicates the mean number of donor axons in the buccal branch in the control group (1685 ± 126). The mean number of myelinated axons of the occipital nerves in the control group was 524 ± 126. The axon counts of both experimental groups are higher than the mean number of donor axons, indicating the presence of axonal sprouting in both groups.

**DISCUSSION**

Our study shows for the first time that motor nerve regeneration through long nerve grafts can be improved by end-to-side coaptation of sensory nerves along the graft. Clinically, this is especially important for facial reanimation, as exceptionally long nerve grafts with lengths of 25 to 30 cm are often used, and functional outcomes are often insufficient.13,14

**Chronic Axotomy and Chronic Denervation Inhibit Axonal Regeneration through Long Nerve Grafts**

Regeneration after peripheral nerve injury is incomplete because of long regeneration times and distances, even if immediate repair is possible.17 With increased regeneration distances, injured neurons progressively fail to regenerate their axons (chronic axotomy), and the denervated distal nerve loses the capacity to support regeneration (chronic denervation).17,37–42 Poor regeneration correlates with the decline of growth factor expression in the denervated nerve stump over time.19,43,44 Increased denervation times reduce the
regenerative capacity of the distal nerve stump: after 6 months of chronic denervation, only 10 percent of axons of acutely axotomized axons regenerate through the distal stump.40

**Protection or Babysitting of Chronically Denervated Nerves Improves Regeneration through Endogenous Trophic Support**

Because of their length, cross-face nerve grafts undergo chronic denervation. In facial reanimation, “babysitter” procedures have been introduced, where a partial hypoglossal-to-facial nerve transfer is performed in patients with facial nerve denervation exceeding 6 months.45–47 The side effects of partial hypoglossal transfer include donor nerve deficits and overpowering of the impulses of the contralateral facial nerve branch, which regenerate through cross-face nerve grafts.48–50 Babysitting or protection of a nerve is based on the concept of providing endogenous growth support by introducing additional sensory or motor (donor) axons into the distal denervated nerve.51–53 Sensory protection as described by Bain et al. improves functional outcomes. In this approach, the transected end of a sensory nerve is coapted to the end of a transected motor nerve to temporarily innervate denervated muscle before the motor repair, even though no excitable neuromuscular junctions are formed.51,52 Ladak et al. described another form of “protection” of denervated distal nerve stumps using side-to-side bridges from a healthy donor nerve to improve regeneration without creating a donor nerve deficit.53 A small number of donor axons regenerating through the side-to-side bridges keep the denervated Schwann cells of the distal nerve stump in a growth-supportive state.51 In our study, we aimed to “protect” the pathway of a denervated cross-face nerve graft with end-to-side addition of small adjacent sensory nerves and to subsequently enhance facial nerve motor regeneration through the cross-face nerve graft. End-to-side neurorrhaphy52–54 is clinically applied in facial reanimation and studied in various animal models.54–57 A modification is the “reverse” end-to-side neurorrhaphy, where the end of the donor nerve is coapted end-to-side to an injured recipient nerve.58 This technique is described as “supercharging” or “up-grading” of nerve function, as the intent is to preserve the regenerated (partial) function of the recipient nerve and augment it through the end-to-side coaptation.6,59–61 By end-to-side coaptation of sensory nerves to the graft we sought to (1) avoid a motor donor nerve deficit and (2) enhance regeneration of facial nerve axons through the cross-face nerve graft to improve the degree of facial reanimation without introduction of another motor donor nerve, which could overpower the facial nerve impulses.

The improvement of whisker motion supports the histologic findings of enhanced regeneration. We used the video analysis technique described by Guntinas-Lichius et al.28,29 which is less invasive compared with other techniques that require the implantation of a fixation plate into the skull and allow for simultaneous analysis of whisker motion and eyelid blinking.62,63 Head immobilization was not necessary for our study, because eyelid closure was not impaired in our model and only whisker motion was studied. Sensory pathway protection of cross-face nerve grafts improved the amplitude of whisking, implying that patients may experience greater excursion during animation were this technique translated clinically. The other whisker movements were not significantly influenced, nor would we have expected them to be, because the medulla’s central pattern generator responsible for whisking rate64,65 was not altered by the sensory protection of the cross-face nerve graft. It was previously shown that the vibrissae motor cortex (M1) controls only the amplitude of the whisk cycles,64 whereas multiple brainstem structures are involved in motion pattern control.65

**In Vivo Imaging of Improved Axon Regeneration through Cross-Face Nerve Grafts in the Thy1–Green Fluorescent Protein Rat**

In vivo imaging of regenerating green fluorescent protein–positive axons in the transgenic Thy1–green fluorescent protein rat model allows for direct serial live imaging over clinically relevant distances.23–25 The design of the surgical model was strongly influenced by the requirements of in vivo imaging of nerve regeneration. The cross-face nerve graft was placed over the forehead,53 to allow for convenient reexposure of the nerve graft for imaging. The in vivo imaging of green fluorescent protein–positive axon regeneration demonstrated that axons reached the recipient side of the face earlier than in the unprotected group. In humans, the regeneration distances are even longer, leading to the possibility that pathway protection of cross-face nerve grafts could play an even greater relative role in improving outcomes.

**Clinical Implications of Protection of Cross-Face Nerve Grafts**

One question facing the future of facial reanimation is whether the regeneration through cross-face nerve grafts can be improved to compete
with the strong results achieved with other donor nerves.\textsuperscript{67–69} Even though the amplitude of motion provided with alternate donor nerves, such as the trigeminal or hypoglossal, is high, and cortical plasticity can occur,\textsuperscript{68} the goal in all cases of facial paralysis should be emotionally spontaneous facial reanimation whenever possible. The sensory pathway protection of cross-face nerve grafts provides a promising option to improve axonal regeneration without creating a motor donor nerve deficit. One could envision a clinical strategy in which axons from adjacent sensory nerves, such as the infraorbital nerves or great auricular nerves, could be directed into the side of the cross-face nerve graft by short sural nerve grafts remaining from the harvest of the cross-face nerve graft. In such a fashion, this experimental strategy could be clinically feasible.

CONCLUSIONS

Nerve regeneration through long nerve grafts, such as cross-face nerve grafts, is often insufficient because of the long distance and duration required for neurons to regenerate their axons across the graft. Protection of the long nerve graft with end-to-side coaptation of small adjacent sensory nerves, which counteracts the chronic denervation of the grafts, improves histologic and functional outcomes. Sensory protection of cross-face nerve grafts could potentially improve the clinical outcomes of facial reanimation.

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