Recurrent Laryngeal Nerve Transection and Anastomosis: Rat Laryngeal Motoneuron Survival and Effect of the Anastomosis Site

Michael J. Pitman, MD; Craig Berzofsky, MD; Opeyemi Alli; Sansar Sharma, PhD

Objectives: We investigated the quantity of recurrent laryngeal nerve motoneurons (RLNMs) that survive after transection and anastomosis of the rat recurrent laryngeal nerve (RLN), as well as the impact of the anastomosis site on RLN regeneration.

Methods: Ten rats underwent right RLN transection and anastomosis. After 16 weeks, Fluoro-Ruby (FR) was applied to the RLN that was transected proximal or distal to the anastomosis site. The brain stems were harvested, and the nucleus ambiguus was evaluated for labeled RLNMs. The RLNM counts were compared to each other and to those from 3 control rats in which FR was applied to an acutely transected RLN.

Results: The number of RLNMs that were stained after RLN transection, anastomosis, and regeneration was consistent with the total number of RLNMs in the nucleus ambiguus of control rats. This finding confirms that most RLNMs survived after RLN transection and anastomosis. The quantity of labeled RLNMs was statistically similar whether the FR was applied proximal or distal to the anastomosis, implying that most of the viable axons that were present proximal to the anastomosis crossed into the distal nerve.

Conclusions: Rat RLNMs survive nerve transection, anastomosis, and regeneration. The anastomosis site does not significantly impede axonal regeneration, and most of the axons traverse the anastomosis into the distal nerve.

Key Words: laryngeal motoneuron, nerve reinnervation, rat model, recurrent laryngeal nerve, vocal cord paralysis, vocal fold paralysis.

INTRODUCTION

Recurrent laryngeal nerve (RLN) injury with vocal fold paralysis, either unilateral or bilateral, is a clinical problem that occurs frequently and results in significant morbidity. Currently, our best rehabilitative operations are static procedures that result in fixed medialization of the vocal fold in patients with unilateral paralysis or tissue destruction and voice deterioration in patients with bilateral paralysis. Optimal treatment would entail functional nonsynkinetic RLN reinnervation resulting in restoration of full vocal fold motion. To that end, many investigators have utilized the rat animal model of RLN injury to study the RLN, recurrent laryngeal nerve motoneurons (RLNMs) of the nucleus ambiguus, and patterns of laryngeal muscle innervation and reinnervation following RLN or vagal injury.1-16

Some of these studies have focused on the anatomy of the RLN and RLNMs, as well as changes in rats after RLN injury without therapeutic intervention.4,8-16 Some have attempted to enhance axonal regrowth with the goal of increasing the rate and number of viable axons regenerating and contacting the motor end plates.1,2 Others have focused on evaluating or preventing RLNM death after RLN or vagal nerve injury.3,5-7

Our experience with the rat model of RLN injury has led us to believe that reinnervation after injury, transection, and anastomosis, without any type of intervention, is robust.4,10,11 It appears that after maturation of reinnervation at 16 weeks after transection and anastomosis, the nerve distal to the anastomosis is histologically similar to a noninjured RLN and the electromyographic (EMG) recruitment is full, albeit synkinetic.4 This result appears to parallel what occurs in humans after RLN injury, as EMG...

From the Department of Otolaryngology, Voice and Swallowing Institute, New York Eye and Ear Infirmary, New York (Pitman, Berzofsky), and the Department of Cell Biology, New York Medical College, Valhalla (Alli, Sharma), New York. This research was supported by the Triological Society Career Development Grant. This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of New York Medical College.

Presented at the meeting of the American Broncho-Esophagological Association, San Diego, California, April 18-19, 2012.

Correspondence: Michael J. Pitman, MD, 310 E 14th St, 6th Floor, New York Eye and Ear Infirmary, New York, NY 10003.
silence after the RLN has had time to reinnervate would be unique. Clinical and scientific evidence suggests that human RLN reinnervation is generally robust after injury and that it is synkinesis that results in poor vocal fold function — not insufficient reinnervation.\textsuperscript{17,18} Considering these observations, we developed and tested two hypotheses: 1) that RLNM\textsubscript{s} survive RLN transection and anastomosis and 2) that significant numbers of regenerating axons consistently cross the anastomosis to occupy the distal nerve. If confirmed, these hypotheses suggest that RLNM survival and robust axonal regeneration across the anastomosis are not significant barriers to reinnervation. These findings would allow research efforts to be concentrated on nerve guidance leading to functional, nonsynkinetic reinnervation.

METHODS

Experimental Animals. Thirteen female Sprague Dawley rats (Charles River Laboratories, Kingston, New York) weighing 250 g were used in the present study. Humane care was provided for the animals, and all institutional (New York Medical College) and national animal care guidelines were observed.

Surgical Procedures. All surgeries were performed with an operating microscope (Carl Zeiss AG, Oberkochen, Germany). A vertical midline cervical incision was made from the hyoid bone to the sternum. The right RLN was exposed in the tracheoesophageal groove, dissected from its surrounding fascia, and sharply transected with iridectomy scissors at the seventh tracheal ring. A 0° 4-mm endoscope (Carl Storz, Tuttingen, Germany) fitted with an epiglottis retractor was inserted transorally to visualize the larynx and confirm paralysis of the right vocal fold. The nerve ends were anastomosed by a common suture-free technique utilizing saline solution–hydrated Gelfoam (Pfizer, New York, New York) to avoid the further nerve trauma of suture anastomosis.\textsuperscript{19} Gelfoam was placed beneath the nerve before transection. After the nerve was transected, the ends were separated and reassorted to simulate movement of the nerve ends consistent with the clinical scenario of nerve transection. Gelfoam was then wrapped around the nerve ends to create a firm connection. The superior laryngeal nerve provides collateral innervation to the endolaryngeal musculature in both rats and humans.\textsuperscript{9,15,20} These collateral innervations may interfere with RLN reinnervation, confounding this investigation. To prevent interference, we exposed the superior laryngeal nerve by retraction of the omohyoid muscle, ligated it proximally and distally using small vessel clips (Ethicon, Somerville, New Jersey), and then transected it between the clips.

Sixteen weeks after the transection and anastomosis, the RLN was sharply transected with iridectomy scissors proximal to the anastomosis in a group of 5 animals (proximal group) and distal to the anastomosis in another group of 5 animals (distal group). To identify the nerve segment distal to the anastomosis, we transected the thyroid isthmus and dissected the thyroid lobe off the trachea and transposed it laterally. A pristine distal RLN was then identified posterior-medial to the lobe. To identify the nerve segment proximal to the anastomosis, we identified the RLN in the tracheoesophageal groove, well inferior to the seventh tracheal ring and the fibrosis in the area of the anastomosis. This area was usually just above the thoracic inlet. After the transection, we isolated the proximal nerve stumps from the surrounding fascia and packed them in Gelfoam soaked in 5% Fluoro-Ruby (FR; EMD Millipore, Billerica, Massachusetts) for retrograde labeling of the RLNM\textsubscript{s} in the nucleus ambiguus. This packing was left in place for 5 minutes, and then the cut nerve was thoroughly washed with phosphate buffer solution (PBS) and the area was dried with cotton swabs to minimize contamination of the surrounding tissue by the dye.

On postoperative day 7, the animals were painlessly sacrificed by lethal inhalation of isoflurane.

The control animals had the RLN transection performed in a manner similar to that in the experimental group. After transection, the RLN was labeled with FR with the same protocol used in the experimental groups. After painless sacrifice on postoperative day 7 by lethal inhalation of isoflurane, all of the brain stems were harvested as described below.

Histology. Immediately after sacrifice, the brain stems were harvested and placed for 4 hours in 4% paraformaldehyde in 0.1 mol/L pH 7.2 PBS. The isolated brain stems were then transferred into 30% sucrose in PBS and kept at 4°C until the tissue sank to the bottom of the container. The brain stems were sectioned with a cryostat (Leica Microsystems, Wetzlar, Germany) into 40-\textmu{}m-thick serial axial sections and mounted onto precoated slides. After the sections were allowed to dry overnight, they were irrigated in sterile saline solution to dissolve the optimal cutting temperature medium and mounted with a 1:1 PBS–glycerine solution and cover-slipped. The slides were kept at 4°C in a closed container to avoid light contamination until examination via fluorescent microscopy. The brain stem sections were examined by two blinded viewers using a Zeiss Axiostep epifluorescence microscope (Zeiss, Oberkochen, Germany) with specific absorption and reflection filters.
The slides were systematically examined, and only cells with visible cell bodies containing a nucleus were counted.

**Statistical Analysis.** A t-test was used to compare the numbers of labeled RLNMs in the "proximal" and "distal" groups. A Kruskal-Wallis nonparametric test was used to compare the numbers of labeled RLNMs in the proximal, distal, and control groups.

**RESULTS**

The control rats had 135 to 188 labeled RLNMs (average, 159.7; SD, 26.7). In the rats in which the RLN was transected and labeled proximal to the anastomosis, the number of RLNMs labeled ranged from 138 to 195 (average, 157.4; SD, 22.3). In rats in which the RLN was transected and labeled distal to the anastomosis, the number of RLNMs labeled ranged from 128 to 228 (average, 174.2; SD, 39.4). Statistical analysis suggests that there was not a significant difference between the number of labeled RLNMs in the proximal group and that in the distal group (p = 0.21). No significant differences were noted in the number of labeled RLNMs among those two groups and the controls (p = 0.76).

**DISCUSSION**

Recurrent laryngeal nerve injury and paralysis, whether unilateral or bilateral, carries significant morbidity. The current treatments, although adequate, are suboptimal. The available treatments are static procedures that result in fixed medialization of the vocal fold in patients with unilateral paralysis and tissue destruction and voice deterioration in patients with bilateral vocal fold paralysis. Optimal treatment would result in a dynamic return of functional vocal fold motion.

In pursuit of this goal, numerous investigations of RLN reinnervation in animal models have been performed. These have generally focused on producing robust axonal regrowth with the aim of increasing the number of viable axons that regenerate and contact the motor end plates. Others have focused on evaluating or preventing RLNM death after RLN or vagal nerve injury. Finally, one investigation cited the anastomosis site as an impediment to reinnervation.

In general, motoneuron cell death occurs when the axon injury is near the soma or if the proximal nerve is denied access to trophic factors in the distal nerve. Cell death is rare in mature spinal neurons, but does occur in mature cranial motoneurons. This is especially true in facial and hypoglossal nerve injuries, presumably because of the short distance to the soma. Whether this RLNM cell death occurs after RLN injury and is an impediment to reinnervation is unclear, as there are conflicting research findings. One can surmise that if axons of the RLN are injured near the larynx, the injury would be far enough from the soma that it may survive. In contrast, a high vagal injury is closer to the soma and may be more likely to result in RLNM cell death. In cats, vagal injuries have resulted in significantly worse laryngeal reinnervation than RLN injuries. After vagotomy, in contrast to RLN injury, regenerating axons did not reach the distal RLN, and there was no EMG activity in the laryngeal muscles. Clinical findings support this result, as patients appear to have better reinnervation after an RLN injury than after a vagal injury. In addition, EMG silence is essentially unheard of after an RLN has been given time to regenerate. The reason for these disparities may be the proximity of the vagal injury to the cell body in contrast to a distal RLN injury. Clinical and scientific evidence also suggests that RLN reinnervation in humans is generally robust after an RLN injury and that it is synkinesis that results in poor vocal fold function, rather than insufficient reinnervation. Our previous research using the same rat model used in this study also suggested robust reinnervation with synkineetic reinnervation as the major reason for persistent vocal fold immobility after RLN injury.

Considering the above, one may also conclude that a significant number of RLNMs survive after distal RLN injury, as this is necessary for robust distal reinnervation. In contrast, some studies have suggested that poor labeling of RLNMs after RLN injury is due to RLNM cell death and that this contributes to persistent vocal fold paralysis. Confounding factors were present in some of these studies. Two used intramuscular injection of tracers that are notoriously unreliable, and one used a new staining technique with highly variable results. Hydman et al found no loss of RLNMs up to 1 month after resection of a segment of the RLN, with counts ranging from 110 to 118. Although theirs was an excellent study, it is confounded by the fact that the researchers did not reanastomose the RLN, depriving the soma of important growth factors in the distal nerve. The number of labeled RLNMs they counted, although consistent, is lower than that found in our study and in investigations of the quantity of RLNMs present without RLN injury. In addition, the study only extended 1 month after injury, whereas 16 weeks are required for the reinnervation to mature. The disparities among the findings of previous investigations leads to confusion as to whether RLNMs survive after RLN injury. Clarify-
Consistently evaluating this issue is obviously important to enhancing our knowledge as we strive toward the ultimate goal of functional RLN reinnervation after injury.

In addition to the uncertainty surrounding the survival of RLNMs after RLN injury, the effect of the anastomotic site on reinnervation of the distal segment of the RLN has not been well described. Although the site has been implicated as a barrier to reinnervation, this issue has only been broached by a single RLN study. In that study, Pascual-Fon et al transsected the nerve and placed the cut ends next to each other as a means of anastomosis. At variable time periods, the RLN was then transected distal to the anastomosis site and stained with biotinylated dextran amines (3 kd) — a relatively novel technique at the time. Unfortunately, of 32 rats that survived 90 to 180 days, RLNMs were not labeled at all in 11 animals. In the ones that did show labeling, the quantity of labeled RLNMs counted was too variable that significant statistical analyses could not be performed. As a result, the validity of the findings reported is unclear.

Considering the above, we embarked on the current study to test the hypotheses that RLNMs survive after RLN injury transection and anastomosis and that significant numbers of regenerating axons consistently cross the anastomosis to occupy the nerve distal to the anastomosis. In contrast to previous studies, ours reanastomosed the RLN with Gelfoam after complete transection to simulate the typical clinical scenario and to allow the soma to communicate with the distal axon. In addition, we used FR (a time-tested and reliable retrograde tracer) to label the RLN both proximal and distal to the anastomosis site. This labeling allowed us to study the quantity of surviving RLNMs after reinnervation, as well as evaluate the quantity of axons that were able to successfully cross the anastomosis into the distal nerve.

In this study, the average number of RLNMs labeled 16 weeks after transection and anastomosis was 157.4 (SD, 22.3; range, 138 to 195) after FR was applied proximal to the site of transection and anastomosis. It was 174 (SD, 39.4; range, 128 to 228) after FR was applied distal to the site of transection and anastomosis (see Figure). These numbers are statistically similar to the average number of RLNMs labeled in our control animals (average, 159.7; SD, 26.7; range, 135 to 188; p = 0.76). These numbers are also similar to the quantity of RLNMs labeled without RLN injury in other studies. These results confirm that most of the RLNMs survive 16 weeks after transection and anastomosis of the RLN. As RLN reinnervation has matured by 16 weeks, it can be postulated that further changes in the nucleus ambiguous are unlikely and that RLNMs present at this time will continue to survive.

In addition to RLNM survival, the average number of RLNMs that were labeled after FR was applied distal to the site of transection and anastomosis (average, 174; SD, 39.4; range, 128 to 228) was statistically similar to the average number labeled after FR was applied proximal to the site of transection and anastomosis (average, 157.4; SD, 22.3; range, 138 to 195; p = 0.21). One would expect fewer RLNMs to be labeled after distal application of FR if the anastomosis site inhibited axons from traversing into the distal nerve. These findings suggest that when transection and anastomosis is performed under ideal conditions, fibrosis, small gaps, or other environmental alterations at the anastomosis site do not play a significant role in impeding RLN reinnervation.

CONCLUSIONS

This study clarifies some of the questions surrounding rat RLN survival and the effect of the anastomosis on reinnervation after transection and anastomosis of the rat RLN. It appears that most of the rat RLNMs survive nerve transection, anastomosis, and regeneration. In addition, the anastomosis site does not significantly impede axonal regeneration, as most of the axons traverse the anastomosis into the distal nerve. Because many of the EMG and kinesiologic findings in rats are similar to those found in humans after RLN injury and reinnervation, it is likely that these conclusions are applicable to humans. Finally, in pursuing the ultimate goal of functional RLN reinnervation after injury, these findings allow for future research efforts to be concentrated on areas such as nerve guidance, instead of on increasing the robustness of reinnervation.
REFERENCES


19. Fischer H. A method of suture-free anastomosis of nerve transplantation is being reported; using facial nerve as the example [in German]. Laryngol Rhinol Otol (Stuttg) 1979;58:154-6.


