*The Laryngoscope*


# Blockade of Glial-Derived Neurotrophic Factor in Laryngeal Muscles Promotes Appropriate Reinnervation

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**Objectives/Hypothesis:** Synkinetic reinnervation of the laryngeal muscles is one of the causes of the poor functional recovery after a recurrent laryngeal nerve (RLN) injury. Glial-derived neurotrophic factor (GDNF) is elevated in rat laryngeal muscles during RLN reinnervation. The specific aim of this investigation was to evaluate the effect of anti-GDNF on RLN reinnervation.

**Methods:** Anti-GDNF antibody was injected into the posterior cricoarytenoid (PCA) 3 days following RLN transection and anastomosis. Larynges were harvested at 7, 14, 28, 56, and 112 days post injury (DPI). Prior to sacrifice, the vocal fold mobility was assessed. Immunostaining to identify neuromuscular junctions was used to evaluate the extent of axonal reinnervation of the PCA, lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA).

**Results:** After anti-GDNF injection into PCA, RLN reinnervation in all muscles was altered when compared to the controls. PCA innervation was delayed. At 7 DPI, only a few axons made synapses in the PCA. In contrast, axons prematurely innervated the LTA and MTA when compared to controls. Innervation was similar to controls at 56 and 112 DPI. Vocal fold motion was enhanced in 10 of 24 animals studied.

**Conclusions:** After injection of anti-GDNF into the PCA, early arriving axons bypass the PCA and enter the LTA. Later arriving axons innervate the PCA and MTA. Vocal fold function is improved as compared to controls. Anti-GDNF injection into the PCA influences the pattern of reinnervation and may result in less synkinetic, more functional innervation.

**Key Words:** Glial-derived neurotrophic factor, recurrent laryngeal nerve, reinnervation, nerve injury.

**Level of Evidence:** NA

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## INTRODUCTION

Surgery in the neck and chest may result in injury of the recurrent laryngeal nerve (RLN), with vocal fold paralysis[1,2] causing substantial patient morbidity. In a severe nerve injury or transection, despite robust motor axonal regeneration and laryngeal reinnervation, vocal fold motion and normal phonatory function is not restored.[1,3] Growth factors are known to exert a guiding influence on regenerating axons.[4–6]

In the rat, acute transection of the RLN causes vocal fold paralysis due to denervation of the intrinsic laryngeal muscles.[7–14] Following injury, the muscle and nerve enter a regenerative state. Axons from the intact proximal end of the nerve sprout and extend toward the larynx.[9–11,13,14] However, regenerating axons are misrouted, leading to nonselective synkinetic reinnervation of the laryngeal muscles. Despite this reinnervation, functional vocal fold motion is never restored.[7,9–11,13,14]

Certain growth factors, like glial-derived neurotrophic factor (GDNF) are upregulated during the regenerating process and may influence reinnervation via axonal guidance of RLN axons.[14,15] GDNF is a member of the transforming growth factor superfamily.[16] Its role during development is to enhance neuron survival.[17,18] GDNF is also expressed within muscles and plays a critical role in neuromuscular junction formation and maintenance.[19–21] Before birth, GDNF is upregulated and reaches a peak of its RNA expression that is correlated with hyperinnervation of the motor endplate by supernumerary axons. The excess synapses are eliminated when the expression of GDNF decreases after birth.[19,20] Following a nerve injury, the expression of GDNF is upregulated within the denervated muscles.[22–24] In the rat RLN, GDNF is overexpressed in the denervated muscles during reinnervation[14] and reaches the peak of expression just before regenerating axons innervate the laryngeal muscles. As the motor axons establish neuromuscular synapses, GDNF messenger RNA is downregulated to control levels.[14]

The role of GDNF in RLN axon guidance and reinnervation of the abductor and adductor laryngeal muscles following RLN injury is not fully understood. With the

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TABLE I.
Number of Animals Used in the Study.

| Groups | Time Periods | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 DPI | 7 DPI | 14 DPI | 21 DPI | 28 DPI | 56 DPI | 112 DPI |
| Test of different concentrations (n = 6) | | 6 | | | | | |
| Untreated (no anti-GDNF) (n = 14) | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Treated (anti-GDNF) (n = 24) | | 4 | 4 | 4 | 4 | 4 | 4 |

DPI = days post injury; GDNF = glial-derived neurotrophic factor.

ultimate goal of influencing RLN regeneration and stimulating nonsynkinetic reinnervation with improved vocal fold function, this study aimed to evaluate the effect of anti-GDNF on reinnervation after RLN transection.

## MATERIALS AND METHODS

### *Experimental Animals*

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act (7 U.S.C. et seq.). The Animal Care and Use Committee of New York Medical College approved the protocol. Forty-four adult female Sprague Dawley rats (250–300 g) were used in the present study (Table I). Thirty-six were grouped based on duration of survival days post injury (DPI). Animals were euthanized on 7, 14, 21, 28, 56, and 112 DPI with an overdose of isoflurane inhalation. In each group, four animals had anti-GDNF antibodies injection (treated), and two had an RLN transection and anastomosis but were not treated with anti-GDNF antibodies (untreated). Two more animals were used as controls to identify the quantity of motor endplates in the posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA). Six additional animals were used to evaluate effects of different concentrations of anti-GDNF antibodies.

### *Surgery, RLN Section, and Repair*

Experimental rats were deeply anesthetized with an intraperitoneal injection of 70 mg/kg of ketamine and 7 mg/kg of xylazine. The larynx was exposed, and the right RLN was identified near the seventh tracheal ring. A piece of Gelfoam (Pharmacia & Upjohn, New York, NY) was gently placed beneath it, and the nerve was transected with an iridectomy scissor. The distal and proximal stumps were aligned with a gap of <1 mm. The ends were then secured by another piece of Gelfoam.

To avoid the collateral innervation to the denervated intrinsic laryngeal muscles from the superior laryngeal nerve,[25] it was identified close to the larynx, ligated, and then transected. Both ends were then separated, and finally, the surgical window was closed, and the animals were returned to their facility with supply of food and water ad libitum.

### *Injection of Anti-GDNF*

Three days after the initial surgery, a second surgery was performed. The surgical window was reopened and the right inferior constrictor muscle was incised and the larynx was slightly rotated to expose the PCA muscle. Two microliters of anti-GDNF antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were slowly injected into the PCA. The injection procedure was performed using a Hamilton Syringe (Hamilton Co., Reno, NV) attached to a micromanipulator on a stereotaxic setup for rat. Following it, a piece of Gelfoam was placed over the injection site in the PCA, and the surgical window was closed.

Several concentrations of anti-GDNF antibodies were tested. Two microliters of 0.5 $\mu$g/mL has no effect on RLN reinnervation at 7 DPI, and 2 $\mu$l of 20 $\mu$g/mL anti-GDNF antibodies showed clear atrophy of the muscle. Two microliters of 2 $\mu$g/mL appeared to effect reinnervation without causing muscle injury and was used for the investigation.

### *Functional Evaluation*

The movement of the vocal folds were observed using a 0°, 4-mm endoscope (Karl Storz, Tuttlingen, Germany), inserted transorally. Movement was recorded by a camera attached to the laryngoscope and a digital recording system (Richard Wolf solid state color video camera; Richard Wolf Medical Instruments Corp., Vernon Hills, IL). The movement of the both vocal folds was evaluated and recorded immediately before the animal was sacrificed. The videos were reviewed for blinded grading of motion of the right vocal fold by one of the authors (M.J.P.): 0 = immobile; 1 = slight nonpurposeful motion, 2 = purposeful but paretic motion, 3 = normal motion[26]. Twenty of 24 animals were recorded for fold movements.

### *Immunohistochemistry*

The animals were euthanized with isoflurane inhalation. Isolated larynges were fixed for 2 hours with 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.1). Later, they were immersed in 30% sucrose in PBS and sectioned in a Leica cryostat (Leica Microsystems, Wetzlar, Germany).

To observe motor axons and motor endplates, immunostaining was performed. Fourteen micrometer–serial sections containing PCA, LTA, and MTA were incubated with rabbit anti-$\beta$-tubulin III polyclonal antibody (1:1000; Covance, Princeton, NJ) in Tris-buffered saline plus 0.8% bovine serum albumin for 48 hours at 4°C. After washing, the sections were incubated in Cy3 conjugated anti-rabbit immunoglobulin G (1:400, Jackson ImmunoResearch, West Grove, PA) for 1 hour at 4°C. The sections were then incubated in AlexaFluor 488 conjugated $\alpha$-bungarotoxin (1:500) for 2 hours at room temperature. Sections were mounted, with glycerin-PBS 1:1 solution, cover slipped, and evaluated.

To establish the total number of motor endplates present in the adult rat control muscles, immunostaining as described above for $\alpha$-bungarotoxin was performed in serial sections of the bilateral PCA, LTA, and MTA dissected out from the larynges of two animals. A total of four muscles per group were used to determine the average number of motor endplates in laryngeal muscles. In experimental animals, motor endplates for each muscle were counted (15% of controls). This amount was considered sufficient so findings may be extrapolated to accurately represent all the motor endplates of a given muscle.[21] Endplates were considered innervated if the images of red-stained axons

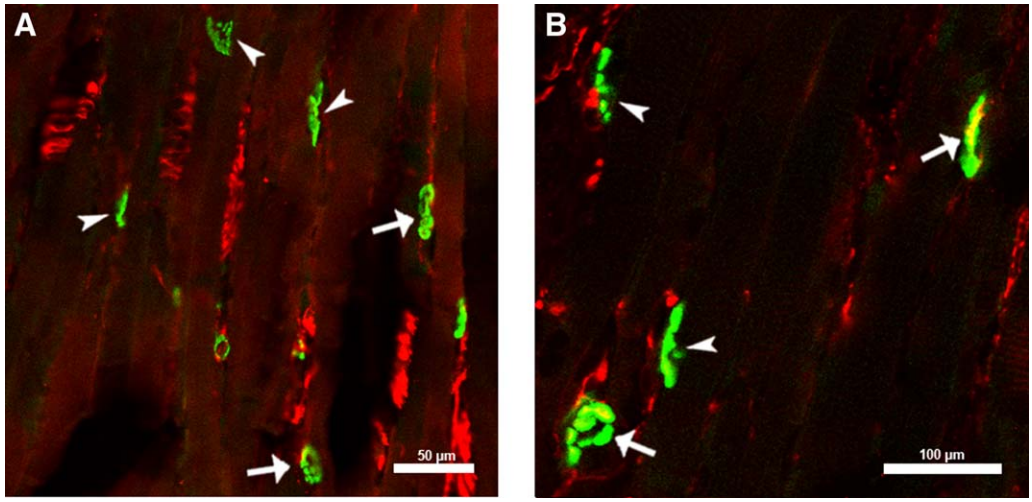


Fig. 1. Confocal images showing labeled motor endplates in green (white head arrows) and motor axons in red in the posterior cricoarytenoid following right recurrent laryngeal nerve injury. Motor endplates innervated by motor axons merged to form yellow (white arrows). At 7 days post injury (DPI) (A), some axons were making synapsis (merge in yellow) in denervated motor endplates. At 21 DPI (B), most of motor endplates were innervated, and only a few were still denervated.

and green-stained motor endplates merged to produce yellow overlapped during image processing. A green motor endplate was deemed uninnervated (Fig. 1). Innervated and uninnervated motor endplates were counted and recorded to establish the percentage of innervation following RLN injury. For each section, the presence and position of axons was noted. Two animals without anti-GDNF antibodies and four animals treated with anti-GDNF antibodies per time point were analyzed. Results are expressed in percentage of innervated motor endplates as compared to the corresponding control muscle. Student *t* test was used to compare treated to untreated reinnervation at different time points following RLN injury.

## RESULTS

### *Vocal Fold Motion Evaluation*

All control animals (without anti-GDNF antibodies) had no vocal fold motion (grade 0) except for one at 21 DPI, two at 28 DPI, and one at 56 DPI. These were graded at as 1 due to indefinable nonpurposeful motion. It is difficult to distinguish between active movement initiated by the vocal fold from passive movement of a paralyzed vocal fold due to activity of the contralateral vocal fold or surrounding musculature. Following injection of anti-GDNF at 7 DPI, no vocal fold motion was present in the right vocal fold. Two experimental animals showed slight fold movement at 14 DPI graded as 1 and 2. At 21 DPI, two of the four animals with adequate videos clearly showed enhanced motion with grades of 2. One animal had a grade of 3, being consistent with normal symmetric vocal fold motion. At 28 DPI, two animals were graded at 2. At 56 DPI, one had grade 2 motion whereas another one was graded at 1. At 112 DPI, all animals had paralyzed vocal folds except one with grade 1.

### *Immunohistochemistry*

The total number of motor endplates in the PCA, LTA, and MTA of control animals were 445 ± 25 in the PCA, 607 ± 26 in the LTA, and 276 ± 39 in the MTA. No differences were observed between left and right side.

Quantification of motor endplate reinnervation in untreated experimental animals corroborates the description of the laryngeal reinnervation.[14] At 7 DPI, RLN axons were found in the PCA establishing synapses to the motor endplates (68% ± 5%) (Figs. 2A and 3A). In contrast, hardly any axons were observed in the LTA and MTA (2% ± 3% and 0%) (Fig. 2E,I and Fig. 3B,C). Motor axons and nerve-muscle synapses were clearly observed at 14 DPI in the LTA (57% ± 1%) (Fig. 2G and Fig. 3B) and at 21 days in the MTA (88% ± 10%). From 21 DPI onward, the motor axon arrangement in the PCA, LTA, and MTA were progressively reorganized into the center of the muscle, similar to the contralateral uninjured side. The percentage of motor endplates innervated from 14 to 112 DPI was close to sham animals in the PCA and LTA, and no significant differences were observed. In the MTA, the percentage of innervation was similar to controls from 21 to 112 DPI.

Following injection of anti-GDNF antibodies into the PCA, several axons were observed in the PCA at 7 DPI; however, in comparison to controls, only a few contacts with motor endplates were observed in the controls (11% ± 5%: $P < .001$, *t* test: Fig. 1A, Fig. 2, and Fig. 3A). In contrast to both the PCA and untreated LTA, a significant number of labeled axons formed neuromuscular synapses with the LTA motor endplates by 7 DPI (54% ± 1%) ($P < .001$) (Fig. 2 and Fig. 3B). Similarly, the MTA in treated animals was innervated in a shorter time period than untreated animals, with the majority of innervation occurring by 14 DPI ($P < .002$) (Fig. 2 and Fig. 3C). Although the arrangement of axons was more immature compared to untreated animals, most of the motor endplates were innervated in the PCA (76% ± 2%), LTA (67% ± 2%), and MTA (70% ± 3%) (Fig. 2D,H,L and Fig. 3).

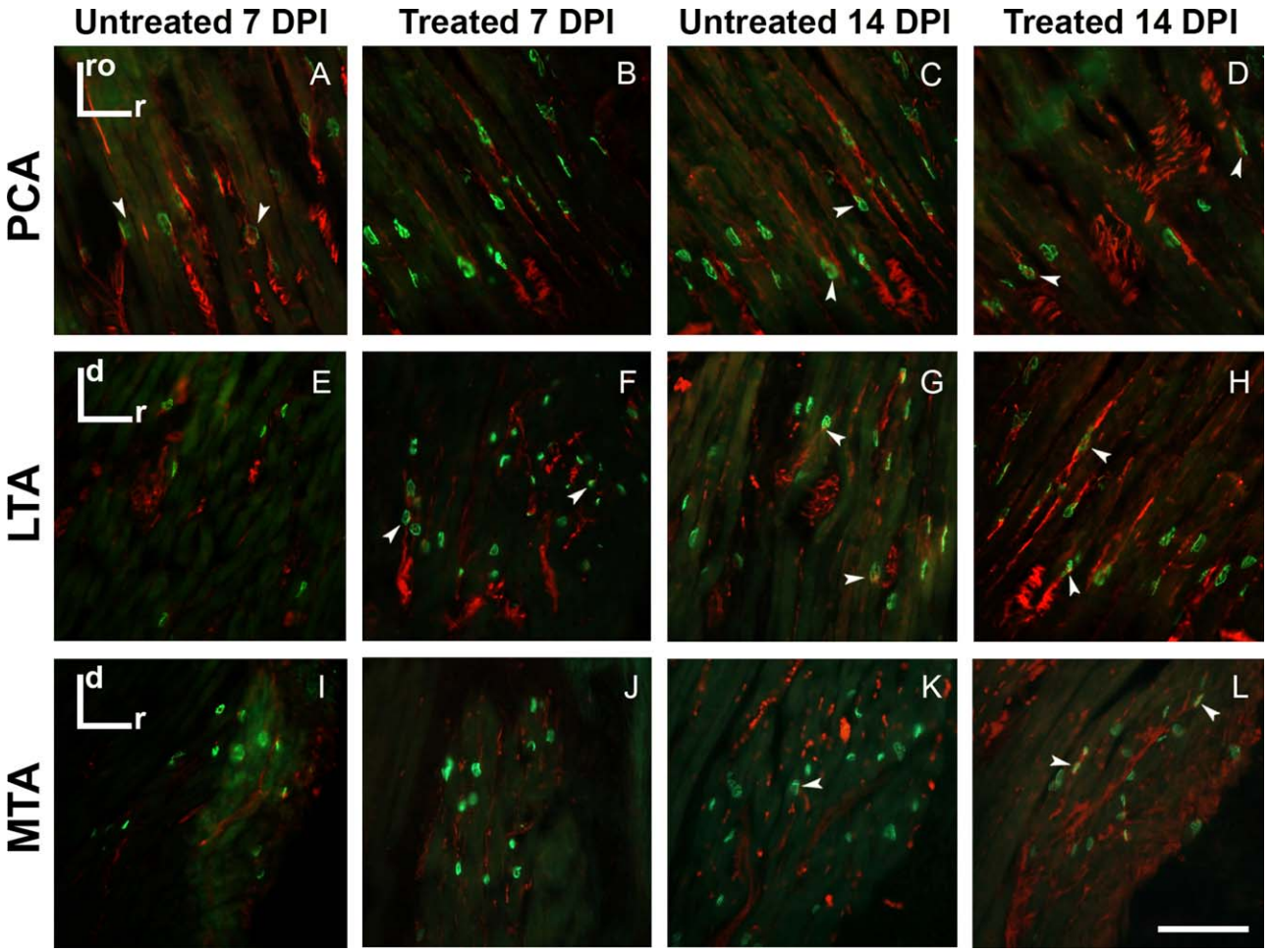


Fig. 2. Labeling of the motor endplates in green and the axons in red in the PCA (A–D), LTA (E–H), and MTA (I–L) at different time points following RLN injury. Motor endplates innervated by motor axons merged in yellow. At 7 days post lesion of the RLN in the PCA (A), some motor axons made synapses (white arrows). In the LTA (E) and MTA (I), motor endplates were labeled, but no axonal labeling was detected. However, following injection of anti-GDNF antibodies, no axons established neuromuscular junction in the PCA (B) and MTA (J), but a significant number of axons were present in the LTA (F). At 14 DPI, many motor endplates are innervated in the PCA (C), but only few in the LTA (G), and not one in the MTA (K). In contrast, several neuromuscular junctions were observed in the PCA (D) and LTA (H) at 14 DPI following injection of anti-GNDF antibodies. Motor endplates were also innervated in the MTA (L). The scale bar in image L = 100 $\mu$m. The remaining images are presented at the same magnification. d = dorsal; DPI = days post injury; GDNF = glial-derived neurotrophic factor; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; PCA = posterior cricoarytenoid; r = right; RLN = recurrent laryngeal nerve; ro = rostral.

From 21 DPI onward, the reinnervation process was steady and similar to untreated animals, with more than 80% of motor endplates reinnervated in both treated and untreated animals (Fig. 1B and Fig. 3). As the neuromuscular junctions matured by 56 DPI, the distribution of motor axons and neuromuscular innervation became similar to controls.

## DISCUSSION

GDNF plays several roles in developing adult muscles in animals. It participates in the formation of the neuromuscular junctions.[19–21,27,28] An upregulation of GDNF promotes the hyperinnervation of motor endplates by several axons during development. Birth triggers the downregulation of GDNF to a basal levels, resulting in the elimination of the supernumerary axons to a single axon per motor endplate.[19–21] Furthermore, early depletion of GDNF expression leads to motor innervation deficits during development.[27,28] In adult muscles, neuromuscular synapse maintenance is supported by the expression of GDNF via regulation of acetylcholine release to the postsynaptic membrane at the neuromuscular junction.[29–31] Following peripheral nerve injury, GDNF encourages polyinnervation in the adult mouse during reinnervation.[32] GDNF is also linked to axon sprouting and guidance after nerve injury in the adult animal.[23,24,32,33] In the rat larynx muscles, levels of GDNF expression change dramatically following RLN injury.[14] The level of GDNF in all denervated laryngeal muscle increases 7 DPI. Once the axons reinnervate the laryngeal muscles, the level of GDNF expression is downregulated. Previous findings and the results of the present study show that axons reach the PCA first, followed by the LTA, and then the MTA. GDNF downregulation consistently follows this progression and is chronologically correlated to the timing of reinnervation. This correlation

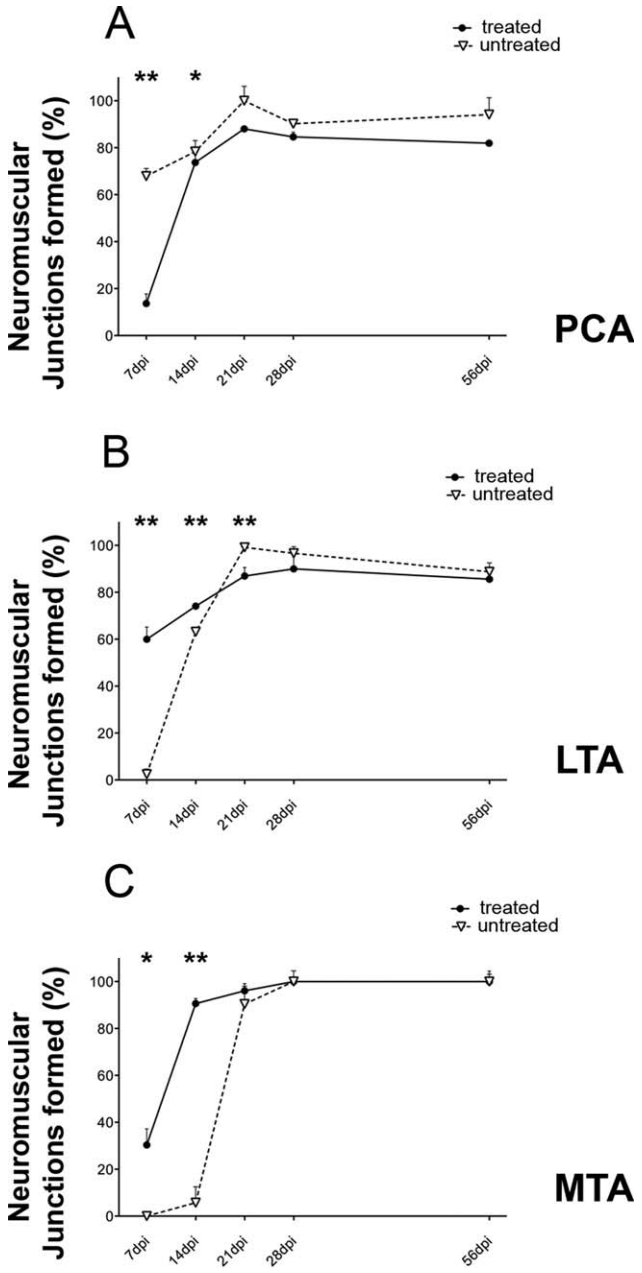


Fig. 3. Number of neuromuscular junctions formed following RLN injury in the PCA (A), LTA (B), and MTA (C) at different time points. Comparative innervation in denervated laryngeal muscles were analyzed. Dashed lines represent proportion of neuromuscular junction formations following the RLN, and the continuous line represents the reinnervation after anti-GDNF antibodies injection into the PCA. In controls, the PCA was the first muscle to be reinnervated, followed by the LTA and MTA. In contrast, injection of anti-GDNF antibodies altered the present pattern. The LTA was reinnervated first at 7 DPI (B), followed by the PCA (A) and MTA at 21 DPI (C). *Results are significantly different; $P < .05$. **$P < .01$. DPI = days post injury; GDNF = glial-derived neurotrophic factor; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; PCA = posterior cricoarytenoid; RLN = recurrent laryngeal nerve.

between reinnervation and GDNF levels in laryngeal muscles, as well as the importance of GDNF to neuromuscular junction formation, axon sprouting, and axon guidance during development and during reinnervation in mature animals, suggests that GDNF overexpression may be linked to axon guidance and the establishment of neuromuscular junctions in laryngeal reinnervation.

Our present results support this hypothesis, as blocking GDNF expression with an injection of anti-GDNF antibodies into the PCA 3 DPI influenced the pattern of reinnervation. In untreated animals, axons enter the PCA first at 7 days following acute nerve injury and they then innervate the LTA and MTA at 14 and 21 days, respectively.[13,14] Similarly, in development, innervation of the PCA occurs first.[34] However, following a single injection of anti-GDNF antibodies into the PCA at 3 DPI, the LTA was clearly reinnervated first. Both the LTA and MTA were innervated early, with significantly increased innervation at 7 and 14 DPI ($P_{\text{LTA 7 DPI}} < .001$, $P_{\text{LTA 14 DPI}} < .008$, $P_{\text{MTA 7 DPI}} < .016$, $P_{\text{MTA 14 DPI}} < .002$). The PCA did not reach a density of innervation consistent with controls until 21 DPI, with decreased density at 7 and 14 DPI ($P_{\text{PCA 7 DPI}} < .001$, $P_{\text{PCA 14 DPI}} < .03$). This change in the pattern of reinnervation may be correlated to the presence of substantial vocal fold motion in seven of 24 experimental animals. Of the seven experimental animals, six had clear purposeful vocal fold motion, whereas one had completely normal and symmetric motion. During early reinnervation, motion in the vocal folds was possibly due to an imbalance of reinnervation favoring the LTA and MTA, with less innervation of the PCA, or may have been due to less synkinetic reinnervation. At 28, 56, and 112 DPI, the amount of reinnervation is the same as controls, so movement at these periods may be solely due to decreased synkinesis, stimulated by the effects of anti-GDNF antibodies. It is possible that the chronologic changes in innervation are accompanied by changes in the pattern of reinnervation, with early reinnervating adductor axons bypassing the PCA that they would have normally innervated in an untreated muscle. With a critical number of axons being guided back to the correct muscle, there may be less synkinesis and restoration of vocal fold motion. If anti-GDNF antibodies sufficiently inhibited the function of GDNF in the PCA, the ability of GDNF to guide axons to the PCA may have been impeded. In addition, axons that were able to enter the PCA may have had difficulty in creating and sustaining neuromuscular junctions due to the dearth of functional GDNF.[32] As a result, axons that arrived first and would normally route to the PCA, may have continued along to the LTA where they established neuromuscular junctions. Similarly, once the anti-GDNF antibodies were no longer present, slow-growing axons that would normally route to the LTA and MTA were able to enter the PCA and form neuromuscular junctions. Further research is necessary to evaluate these hypotheses. Specific studies may entail investigation of GDNF's ability to guide axons and influence neuromuscular junction formation in the larynx, differences in the response between adductor and abductor axons, and the changes in synkinetic reinnervation stimulated by the PCA injections of anti-GDNF antibodies.

One significant confounding factor in this study is the limited exposure of the PCA to anti-GDNF antibodies. There is likely a limit to the functionality of the anti-GDNF antibody when it is injected once at 3 DPI.

Further investigations involving multiple injection time points and the slow release of anti-GDNF antibodies into laryngeal muscles are essential. The results of the present study encourage further research as neurotrophic growth factors may represent a promising treatment by inhibiting synkinesis and restoring vocal fold function following RLN injury.

## CONCLUSION

Following a RLN injury, the reinnervation process of axonal guidance and the reformation of neuromuscular junctions are influenced by the expression of GDNF. A single injection of anti-GDNF antibodies into the PCA resulted in an altered pattern of reinnervation of the laryngeal muscles. Contrary to controls, where the PCA is the first muscle to be reinnervated followed by the LTA and MTA, injection of anti-GDNF antibodies causes a delay in the formation of synapses in the PCA and accelerates LTA and MTA innervation. Restoration of vocal fold function was enhanced in experimental animals. This occurred also at 56 and 112 DPI, despite axonal reinnervation being dense and appearing histologically similar to untreated groups. These observation suggest that not only was there a change in the chronologic pattern of reinnervation, but that reinnervation was healthier, with less synkinesis, allowing restoration of vocal fold function.

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