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Differential Expression of Glial-Derived Neurotrophic Factor in Rat Laryngeal Muscles During Reinnervation

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Objectives/Hypothesis: Nonspecific, synkinetic reinnervation is one of the causes of poor functional recovery after a peripheral nerve lesion. Knowledge of the differential expression of neurotrophic factors that subserve axon guidance, as well as neuromuscular junction formation and maintenance in the denervated muscles, may allow appropriate interventions that will improve the functional nonsynkinetic reinnervation.

Study Design: Laboratory experiment.

Methods: The expression of glial-derived neurotrophic factor (GDNF) was studied in the abductor and adductor muscles of the larynx in the rat utilizing real-time polymerase chain reaction at different times following transection, anastomosis, and reinnervation of the right recurrent laryngeal nerve (RLN). Immunostaining of GDNF, axons, and the motor endplates were performed. This data was correlated with intramuscular mRNA GDNF expression.

Results: Significant upregulation of GDNF was observed until 14 days after RLN injury. The highest level of the GDNF expression was reached at different times in posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA). These expression peaks correlated with the timing of reinnervation observed on immunohistochemistry, where PCA was reinnervated first, followed by MTA and LTA.

Conclusion: Differences of GDNF expression are linked to the differential timing of RLN axon regeneration and individual muscle reinnervation. The present finding suggests the need to further investigate the role of GDNF and other neurotrophic factors in the timing of reinnervation, axon guidance, and neuromuscular junction formation as it relates to synkinetic and nonsynkinetic RLN reinnervation. Future experimental results may provide insight to therapeutic options that could stimulate appropriate neuromuscular junction formation and nonsynkintic functional reinnervation following RLN injury.

Key Words: GDNF, recurrent laryngeal nerve, reinnervation, nerve injury.

Level of Evidence: N/A.

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INTRODUCTION

Recurrent laryngeal nerve (RLN) injury with resultant vocal fold paralysis results in significant patient morbidity and occasionally mortality. Although there are static surgical procedures for rehabilitation, an optimal treatment that restores normal voice with vocal fold function does not exist. The ultimate goal of stimulating nonsynkinetic reinnervation to restore normal laryngeal function has been elusive. To this end, using the established rat model of RLN injury, this study aimed to investigate the role of Glial-derived neurotrophic factor (GDNF) in RLN reinnervation.

As a consequence of an injury to the RLN in rat, the ipsilateral laryngeal fold becomes paralyzed.[1–9] The injury activates a regenerative state of the nerve with newly sprouting axons from the intact proximal end of the nerve growing toward and reinnervating the denervated laryngeal muscles.[4–6,8,9] Despite the reinnervation, the movement of the vocal folds is never fully restored because of the nonselective synkinetic reinnervation of regenerating axons. The somatotopic map of the laryngeal motoneurons in the nucleus ambiguus, as well as electromyographic data of the laryngeal muscles following regeneration of the RLN, show a strong evidence that the laryngeal motor neurons survive and reinnervate the larynx but that the continued absence of vocal fold motion is due to nonfunctional synkinetic reinnervation.[1,8,9]

GDNF is a member of transforming growth factor-β superfamily.[10] It is has multiple roles in development—in adults and after peripheral nerve injury. During development, GDNF induces spinal motoneurons survival in chicken[11] and rat.[12,13] It is also an important survival factor for motoneurons during development in the chick brainstem.[11,14] During mouse development, GDNF participates in establishing neuromuscular junctions in hindlimb muscles.[15] In postnatal rat, GDNF is continuously produced by skeletal muscle and is transported retrograde to neural somas.[16,17] In adult, GDNF prevents neural death and promotes acetylcholine releases in motor endplates innervated by spinal nerves in mice[18] and cranial nerves in rat.[16,17,19] Following an injury to the sciatic nerve, the levels of GDNF are upregulated in the

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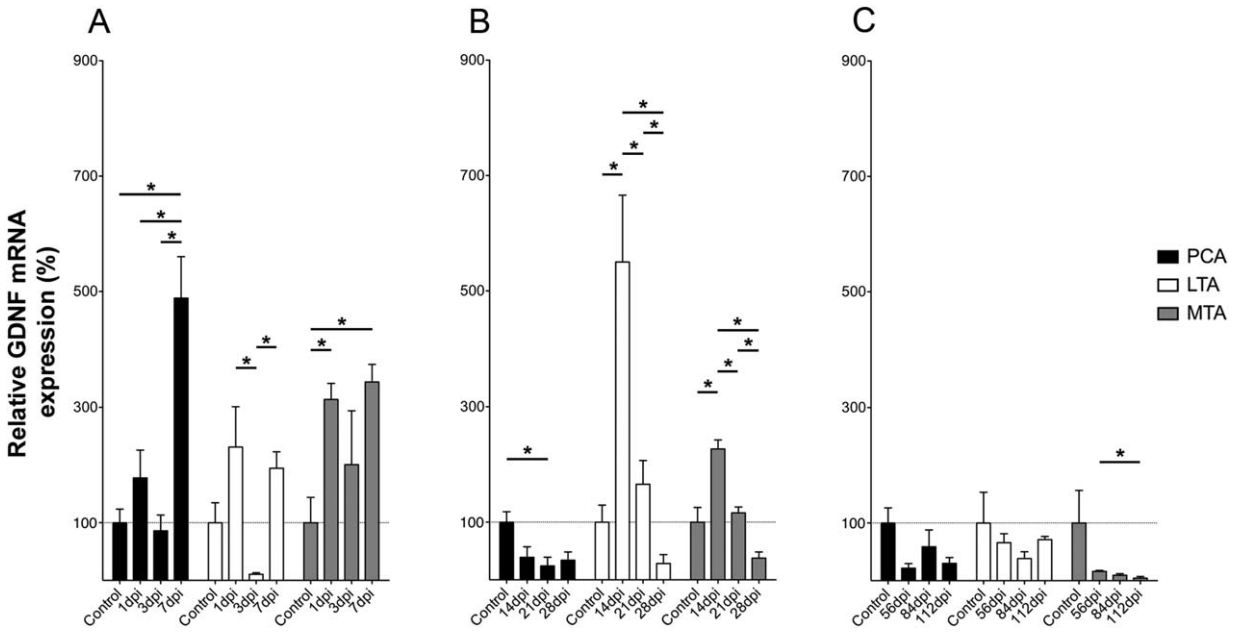

Fig. 1. mRNA expression of GDNF during RLN regeneration at different time points. Comparative expression among PCA, LTA, and MTA from 1 day to 1 week postinjury. (A) 2 to 4 weeks (B) and 8 to 16 weeks (C). Dash line means baseline of control expression. Asterisk (*) indicates results are significantly different, $P < 0.05$. dpi = days post-RLN injury; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; PCA = posterior cricoarytenoid.

gastrocnemius and the soleus muscles of mice[20] and in the gastrocnemius muscle of rat.[21] GDNF upregulation also enhances the neuromuscular polyinnervation of the motor endplates of deep lumbrical muscles during tibial nerve reinnervation in mice.[22]

Realizing the importance of GDNF to muscle innervation during development and in adults, as well as its role after nerve injury, the present study was designed to determine the changes in the expression of GDNF following transection and anastomosis of the RLN. We used mRNA isolated from posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA) from Sprague Dawley rat. Expression of GDNF at different time periods postinjury was studied in abductor (PCA) and adductor muscles (LTA and MTA) performing real-time polymerase chain reaction (RT-PCR). Results were compared between muscles correlated to immunostaining of GDNF, axons, and motor endplate in the same muscle groups at the same time periods.

MATERIALS AND METHODS

Experimental Animals

Eighty-four female Sprague-Dawley rats (250–350 g body weigh) were used in the present study. Animals were anesthetized with an intraperitoneal injection of 70 mg/kg of ketamine and 7 mg/kg of xylazine. The study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.). The Institutional Animal Care and Use Committee of New York Medical College approved the animal use protocol.

Surgery

All surgeries and muscle dissections were performed using an operating microscope (Carl Zeiss, Oberkochen, Germany). A midline skin incision was made in the neck, and the larynx was exposed. The right RLN was identified and transected with iridectomy scissor at the seventh tracheal ring. After the section, the nerve ends were anastomosed using saline hydrated gelfoam (Pharmacia & Upjon, New York, NY). A piece of gelfoam was placed beneath the nerve before the transection. Once the transection was made, the nerve ends were realigned and another piece of gelfoam was placed on top, securing the nerve between the two layers of gelfoam.

In order to avoid SLN confounding collateral innervation to the intrinsic laryngeal muscles during RLN regeneration,[23] the right SLN was transected and both ends were ligated using small vessel clips (Ethicon, Somerville, NJ). The surgical wound was closed in layers. Before the animal recovered from anesthesia, a 0° 4-mm endoscope (Karl Storz, Tuttingen, Germany) was inserted transorally to confirm right vocal-fold paralysis. Another 18 rats divided in three groups were maintained unoperated as control groups.

Tissue Samples and RT-PCR

At 1, 3, 7, 14, 21, 28, 56, 84, 112 days following RLN injury, 54 animals (6 rats per group) were anaesthetized, and evaluation of vocal fold movement was performed as explained above. A similar procedure was performed on 18 uninjured animals used as controls of GDNF expression. Animals were then euthanized with isoflurane inhalation. Right PCA, LTA, and MTA were each carefully dissected and frozen in liquid nitrogen and kept at −80°C.

Total RNA was extracted according to the TRIzol method (Invitrogen, Grand Island, NY). Reverse transcription reactions were performed in a thermo cycler PCR System 2400 (Perkin Elmer, Waltham, MA) using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN).

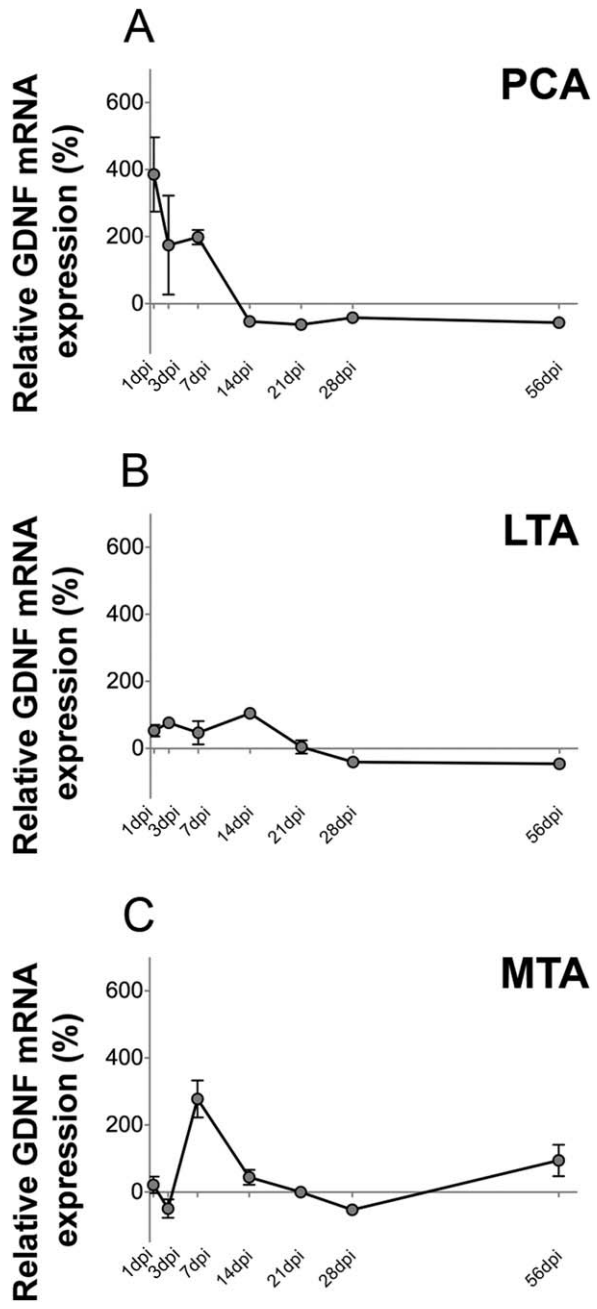

Fig. 2. mRNA expression of GDNF measures in the same plate from 1 day to 8 weeks in posterior cricoarytenoid (A), lateral thyroarytenoid (B), and medial thyroarytenoid (C). dpi = days post-RLN injury; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; PCA = posterior cricoarytenoid.

Two kinds of RT-PCR were performed. In order to compare GDNF mRNA level expression among three muscles, three different RT-PCR plates were made according to time period: 1 to 7 days, 14 to 28 days, and 56 to 112 days. In contrast, to evaluate the changes of GDNF expression in each muscle group along the timeline, RT-PCR plates were constructed to test one muscle group per plate—including specimens 1 to 56 days postinjury.

The relative expression of GDNF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by real-time quantitative RT-PCR using FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN) on an ABI PRISM 7000 Sequence Detection Systems (Life Technologies, Grand Island, NY). The sequence of the primers used were: 5′-GCCGAGGGAGTGGTCTT-3′ (forward), 5′-AGATGAAGTTAT GGGATGTCGTG-3′ (reverse) for GDNF and 5′-TGGACCACCC AGCCCAGCAAG-3′ (forward), 5′-GGCCCCTCCTGTTGTTATG GGGT-3′ (reverse) for GAPDH. They were synthetized by Integrated DNA Technologies (Coralville, IA). For relative quantification of GDNF expression, we used the comparative Ct method ($2^{\Delta Ct}$, where ΔCt represents the differences between GDNF and the internal control gene GAPDH). Prism 5 software (Graphpad Software, La Jolla, CA) was used to preform statistical analysis employing ANOVA and *t* test.

Immunohistochemistry

For evaluation of neuromuscular reinnervation and GDNF expression in specific muscle fibres, 12 rats were used. At 1, 3, 7, 14, 21, and 28 days following RLN injury, rats were euthanized via inhalation of isoflurane (2 animals per time period). Animals were then transcardically perfused with 0.1 M phosphate buffer saline (PBS) (200 ml), followed by 250 ml 4% paraformaldehyde in PBS. The larynx were removed and kept in 30% sucrose in PBS before sectioning them in a Leica cryostat (Leica Microsystems, Germany). Next, 14 μm serial sections of the muscles were collected.

To identify the presence of axons and motor endplates in the muscles, sections were labeled with neuronal class β-tubulin polyclonnal antibody (Covence, Princeton, NJ) and alexa 488 conjugated α-bungarotoxin (Invitrogen, Grand Island, NY), as described.[2] Briefly, after sections were washed with 5% tris-buffered saline (TBS) (0.1 M, pH 7.4), they were transferred to 5% bovine serum albumin (BSA) in TBS for 30 minutes. Sections were incubated with neuronal class β-tubulin polyclonal antibody (dilution 1:1000) in TBS plus 0.8% BSA for 48 hours at 4°C. Sections were then washed twice in TBS with 5% normal sheep serum (NSS) and incubated in anti-rabbit immunoglobulin G (IgG) Cy3 conjugated (1:400 in TBS with 0.8% NSS) for 1 hour at 4°C. Finally, sections were incubated in alexa 488 conjugated α-bungarotoxin (1:500 in 5% TBS) for 2 hours at room temperature.

To determine the localization of GDNF in the laryngeal muscles, sections were washed in PBS and preincubated with 5% donkey serum in PBS plus Triton 0.1% for 30 minutes at room temperature. Sections were then incubated with 1:50 GDNF polyclonal antibody (Santa Cruz Biotechnologies, CA) in Triton 0.1% with PBS plus 0.8% donkey serum overnight at 4°C. After the incubation, slides were washed twice with PBS and incubated in IgG Alexa Fluor 594 conjugate (1:200) for 2 hours at room temperature.

Sections were mounted with glycerin:PBS 1:1 solution, coverslipped, and stored at 4°C. Sections were evaluated using a Zeiss Axioskop epi-fluorescence microscope (Carl Zeiss).

RESULTS

Vocal Fold Motion Evaluation

In all experimental rats, at all time points studied, no movement of the right vocal fold was observed following right RLN transection and anastomosis.

GDNF Expression

RT-PCR of mRNA isolated from ipsilateral laryngeal muscles showed changes in the expression of GDNF following right RLN injury. From 1 to 14 days postinjury,

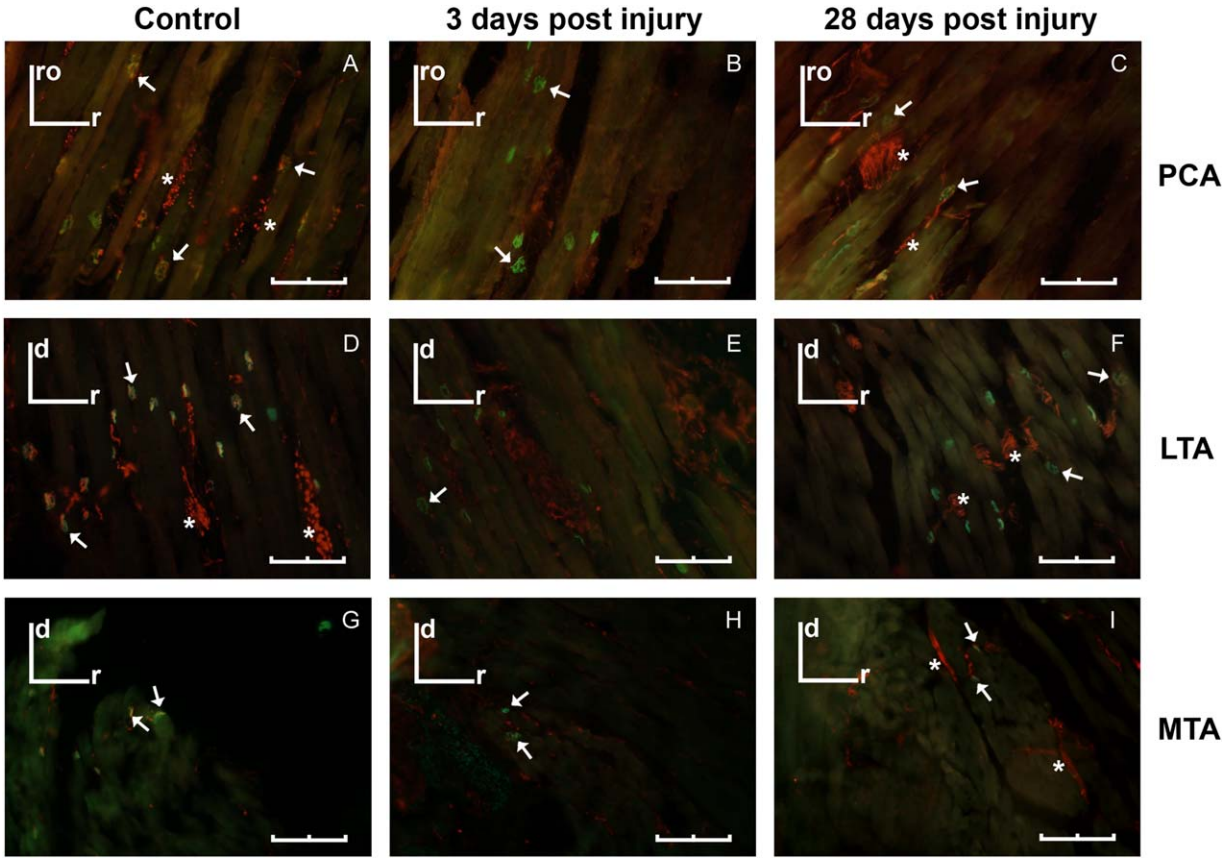

Fig. 3. Labeling of the motor endplates in green (white arrows) and the axons in red (white asterisks) in PCA (A, B, C), LTA (D, E, F), and MTA (G, H, I) at different time points following RLN injury. PCA (A), LTA (D), and MTA (G) of the left side show a positive axonal stainning in the motor endplates. At 3 days postlesion of RLN in PCA (B), LTA (E), and MTA (H), motor endplates were labeled, but no axonal labeling was detected. However, the three muscles (C, F, I) showed the presence of axons and motor endplates at 28 days following right RLN injury. The scale bar in each photograph represents 100 μm. d = dorsal; PCA = posterior cricoarytenoid; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; r = right; ro = rostral.

there was an overexpression of GDNF in all muscles. After 14 days postinjury, GDNF mRNA levels were slightly lower as compared to right PCA, LTA, and MTA in noninjured control animals. ANOVA analysis of the results showed significant differences among three muscles from 1 to 28 days but not from 56 to 112 days following nerve injury.

GDNF expression varied among all three muscles in absolute percentage, although changes relative to the day were more closely linked up to day 7 (Fig. 1). Initially at 1 day postinjury, GDNF expression level increased in PCA, LTA, and MTA. This level then decreased at 3 days postinjury in all muscles. The expression level of GDNF then increased again 7 days postinjury, reaching a peak in PCA and MTA that were significantly relevant compared to control ($P < 0.007$ for PCA; $P < 0.011$ for MTA). The GDNF level expression increased also in LTA, but it was not the peak (Fig. 1 and 2).

At 14 days postinjury, there was a reduction of GDNF expression to below control levels in PCA. In contrast, levels were significantly elevated in LTA and MTA, with LTA reaching a peak (Fig. 1B; Fig. 2B–2C). At 21 days postinjury, the level of GDNF expression in PCA was still below controls; whereas in MTA and LTA it had decreased and was similar to controls. From 28 to 112 days postinjury, the level of GDNF expression in all three muscles was reduced when compared to the control level of GDNF expression—with the exception of one outlier in the MTA on day 56 (Fig. 1B–1C; Fig. 2).

Immunohistochemistry

Following injury to the right RLN, axon and motor endplate immunohistochemical labeling was undertaken as described above. The contralateral laryngeal muscles served as controls. Alexa 488-conjugated α-bungarotoxin labeling of motor endplates was observed in the middle of the muscle belly of PCA, LTA, and MTA. No variation of α-bungarotoxin labeling was found from 1 to 28 days in the denervated muscles (Fig. 3). At 1-day, postinjury β-tubulin–labeled axons were observed in the ipsilateral PCA, LTA, and MTA, but there was evidence of nerve degeneration in the PCA with less intense axon immunoreactivity near the motor endplates when compared to the uninjured side. At 3 days following nerve transection, no

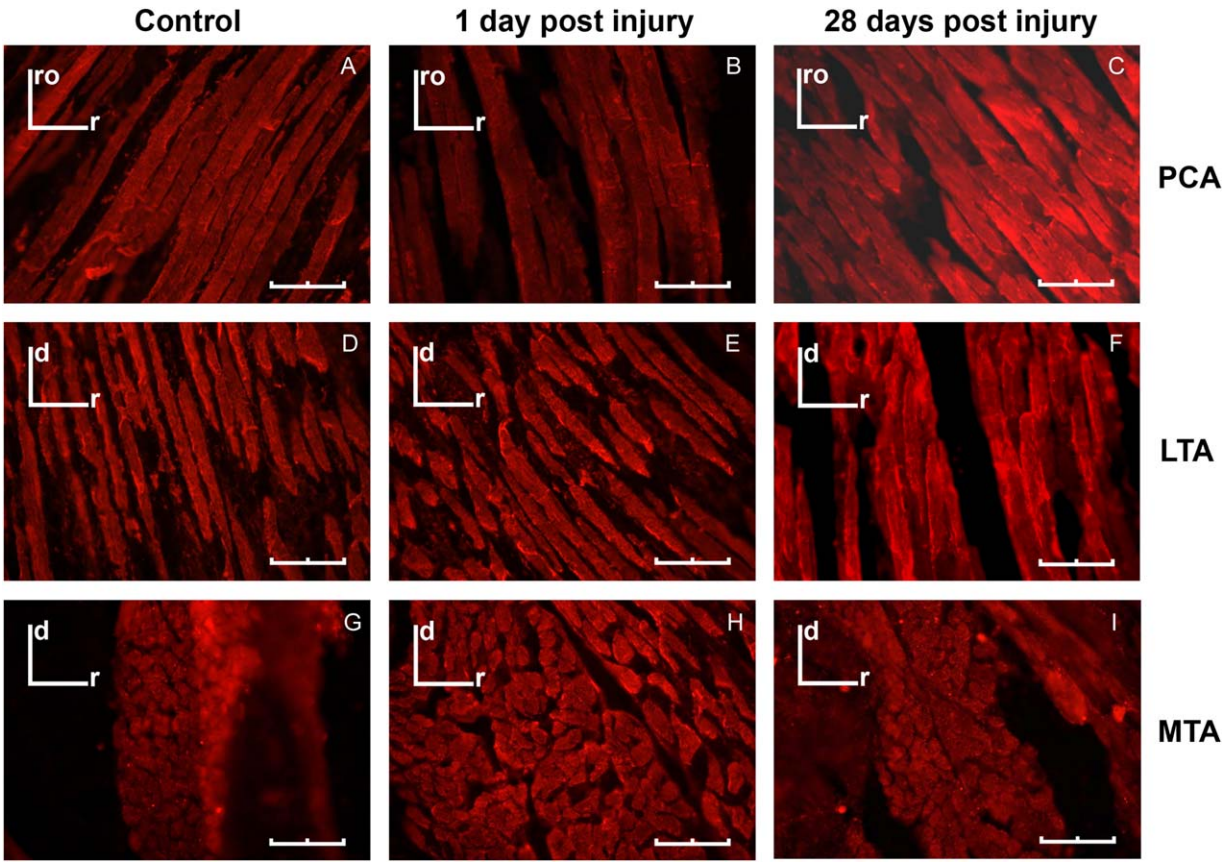

Fig. 4. GDNF labeling of laryngeal muscles at different time points following RLN injury. GDNF labeling was performed in PCA (A, B, C), LTA (D, E, F), and MTA (G, H, I). All staining is seen with the muscle fibers. There was no difference in GDNF labeling pattern between controls and experimental muscles or between any experimental muscle and another experimental muscle. The scale bar in each photograph represents 100 μm. d = dorsal; LTA = lateral thyroarytenoid; MTA = medial thyroarytenoid; PCA = posterior cricoarytenoid; r = right; ro = rostral.

labeled axons was observed in the ipsilateral laryngeal muscles, including the LTA and MTA (Fig. 3B, 3E, 3H). At 7 days postinjury, some axons were labeled in the PCA, but axons were randomly distributed in the muscle. From 14 to 28 days postinjury, the right PCA showed a progressive reorganization of axons similar to the contralateral control PCA (Fig. 3C). At 7 days postinjury, no labeled axon was observed in LTA and in MTA. At 14 days postinjury, a few axons were labeled in LTA as compared to minimal labeling in MTA. At 21 and 28 days postinjury, axonal labeling in ipsilateral LTA and MTA appeared similar to the control pattern (Fig. 3F, 3I).

GDNF immunoreactivity was uniformly distributed within muscle fibre (Fig. 4A, 4D, 4G). No differences were observed between denervated ipsilateral and noninjured contralateral side at any time point studied (Fig. 4).

DISCUSSION

In the present study we showed an upregulation of mRNA GDNF expression in laryngeal muscles, and it was differentially expressed in PCA, LTA and MTA at various time points after RLN transection and reinnervation. GDNF mRNA within laryngeal muscles increases for a short period following a RLN injury. There is an acute rise at day 1 and then a subsequent large decrease on day 3. It is possible that the injured neuromuscular junction may stimulate the upregulation of GDNF at day 1. GDNF has been reported to play such a role in muscles during development. GDNF participates in the formation and maintenance of neuromuscular junctions.[25–27] This maintenance function of GDNF persists in adult muscles because it regulates acetylcholine release to the postsynaptic membrane in neuromuscular junction.[16,17,28] The increased GDNF may be also due to upregulation for retrograde transport to aid in motoneuron survival. In addition, the increased GDNF may be due to decreased efficiency of retrograde transport of GDNF, resulting in its accumulation in the muscles. Comparably, it has been shown that in the severed peripheral nerve, the proximal and the distal ends of the axon become sealed within 2 hours.[29,30] The GDFN that is normally transported retrogradely to the neuron is likely transported toward the distal sealed end of the axon and accumulates there.[16,17,31]

By day 3, Walleran degeneration had occurred, as suggested, by the lack of axons in the experimental

muscles. As a result, the need for neuromuscular junction maintenance and motoneuron survival would be negligible and the need for GDNF would be decreased. Thus, GDNF expression level should be downregulated in the muscles. This assumption is supported by the reduced expression of GDNF observed in the present study after Wallerian degeneration at 3 days following RLN transection.

At 7 days postinjury, the level of GDNF was upregulated in PCA, MTA, and LTA while reinnervating axons approach the muscles. During nerve regeneration, GDNF is known to facilitate the neuronal survival, promote the axonal sprouting of the nerve, and enhance the polyinnervation of motor endplates.[15,25,32–34] We postulate that the GDNF upregulation seen here is due to the critical role of GDNF in inducing axon growth, guiding axons to the muscle, and enhancing the formation of neuromuscular junctions during reinnervation of denervated laryngeal muscles.

After innervation or reinnervation, GDNF is downregulated. During development of sternomastoid and gastrocnemius muscles in mice, GDNF expression was elevated and motor endplates were polyinnervated.[15,25,34] In 1 week postnatal mice, GDNF expression within muscles was downregulated and supernumerary neuromuscular synapses were eliminated.[25,34] This downregulation of GDNF after innervation was observed in the present study. While axon invasion of the PCA begins on day 7 and progresses until day 14, GDNF expression in the PCA goes from a peak to below that of controls. Similarly in the MTA and LTA, while reinnervation occurs between days 14 and 21, GDNF expression in these muscles drops from a peak to a level near controls.

This temporal pattern of rat PCA reinnervation followed by LTA and MTA parallels previous reports. During embryogenesis of the rat larynx,[24] RLN axons reach PCA first, followed by LTA and finally MTA. This pattern of innervation also occurs following RLN transection, anastomosis, and reinnervation in adult rats.[6,9] It is possible that GDNF influences this pattern via axon guidance and neuromuscular junction formation. The drop in the PCA GDNF expression once the PCA is well innervated is correlated with the initiation of axon innervation of the LTA and MTA.

CONCLUSION

Following a RLN transection and regeneration, the expression of GDNF varies among PCA, MTA, and LTA in the rat. These differences correlate with the timing of Wallerian degeneration and then muscle reinnervation, where PCA is the first muscle to be reinnervated followed LTA and MTA. GDNF plays an acute role after axon injury and then in RLN reinnervation, where it may be involved in axon guidance as well as neuromuscular junction formation. Further research investigating GDNF and other neurotrophic factors in PCA, MTA, and LTA after RLN injury and reinnervation will enhance our understanding of the influence of these factors on reinnervation. This knowledge may suggest therapeutic options for nerve guidance, stimulating nonsynkinetic functional reinnervation following RLN injury.

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