


# Recurrent Laryngeal Nerve Reinnervation in Rats Posttransection: Neurotrophic Factor Expression over Time

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## Abstract

**Objective.** Recurrent laryngeal nerve (RLN) injury causes vocal fold paralysis from which functional recovery is typically absent due to nonselective reinnervation. This study investigates expression of axon guidance cues and their modulators relative to the chronology of reinnervation by examining the expression of glial-derived neurotrophic factor (GDNF), netrin 1, and laminin III (LAMA1) in non-pooled laryngeal muscles. This study is the first to describe the post-RLN injury expression pattern of LAMA1, a target of particular interest as it has been shown to switch netrin 1-mediated growth cone attraction to repulsion.

**Study Design.** Animal experiment (rat model).

**Setting.** Basic science laboratory.

**Methods.** The right RLNs of 64 female Sprague-Dawley rats were transected, with sacrifice at 1, 3, 7, 21, 28, and 56 days postinjury (DPI). Single-animal messenger RNA was isolated from the ipsilateral posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA) for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Immunostaining for LAMA1 expression was performed in the same muscles.

**Results.** LAMA1 was elevated in the PCA at 3 to 56 DPI, LTA at 7 DPI, and MTA at 14 and 28 DPI. This correlates with the chronology of laryngeal reinnervation. Using a new protocol, single-animal muscle qRT-PCR possible and expression results for GDNF and netrin 1 were similar to previous pooled investigations.

**Conclusion.** Reliable qRT-PCR is possible with single rat laryngeal muscles. The expression of netrin 1 and LAMA1 is chronologically coordinated with muscle innervation in the LTA and MTA. This suggests that LAMA1 may influence netrin 1 to repel axons and delay LTA and MTA reinnervation.

## Keywords

recurrent laryngeal nerve, nerve injury, reinnervation, GDNF, laminin I, laminin III, netrin I

Injury to the recurrent laryngeal nerve (RLN) is one consequence of thoracic and cervical surgery. The RLN supplies motor innervation to the intrinsic laryngeal muscles. Vocal fold paralysis after RLN insult leads to loss of normal voice, airway obstruction, and possible aspiration.<sup>1,2</sup> Despite robust reinnervation of the denervated laryngeal muscles, newly formed axons are misrouted, resulting in laryngeal synkinesis.<sup>3,4</sup> As a result, vocal fold function is never fully restored, with potentially devastating effects on patients' quality of life. Improved understanding of the molecular mechanisms underlying post-RLN injury may unveil potential therapeutic levers for improving laryngeal muscle reinnervation.

Postinjury expression of several trophic factors has been described, including that of glial-derived neurotrophic factor (GDNF) and netrin 1.<sup>4,5</sup> Cutting and reanastomosing the RLN triggers an upregulation in the expression of these trophic factors within laryngeal muscles at different time points postinjury. GDNF participates in neuron survival and neuromuscular synapse formation during development.<sup>6-9</sup> Upregulation of GDNF expression following RLN injury is correlated with laryngeal muscle reinnervation.<sup>4,10-12</sup>

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Netrin 1 participates in motor axon guidance within the neural tube and toward muscles during development and participates in neuromuscular synapse formation.<sup>13-17</sup> In addition, netrin 1 is a bifunctional axonal guidance cue. It can serve as either an attractive or a repulsive signal, depending on which netrin 1 receptors are expressed on the axonal growth cone.<sup>18-20</sup> Adding another layer of complexity is laminin 111 (LAMA1), a component of the extracellular matrix supporting muscle fibers. LAMA1 has been found to convert netrin 1 into a repulsive signal for the growing axon.<sup>21,22</sup> The role of LAMA1 during laryngeal nerve reinnervation has not been investigated in the larynx. Thus, exploration of the role of LAMA1 in reinnervation is a critical step in elucidating the molecular underpinnings of postinjury synkinesis and, ultimately, how to alter them therapeutically.

Most studies investigating the molecular mechanism of laryngeal reinnervation have been performed in rats.<sup>4,5,23-25</sup> However, the small size of rat laryngeal muscles limits the amount of protein and genetic material that can be isolated from each sample. This can make molecular analysis, like quantitative reverse transcription polymerase chain reaction (qRT-PCR), difficult. It is common to pool laryngeal muscle samples from multiple rats to isolate a sufficient amount of messenger RNA (mRNA) for qRT-PCR analysis.<sup>4,26-30</sup> Disadvantages of this pooling method include the large number of rats needed for a single analysis and inability to study the range of variation within an experimental group. Thus, a protocol that allows for qRT-PCR analysis using less isolated mRNA may increase the strength and reproducibility of the studies published.

In this study, we apply a new method for mRNA isolation and perform qRT-PCR analysis of GDNF, netrin 1, and LAMA1 expression in different laryngeal muscles at various time points following unilateral RLN injury. This is the first study to investigate the temporal expression pattern of LAMA1 after RLN injury. In addition, results for GDNF and netrin 1 are compared to those of previous experiments to assess reliability of the new method.

## Methods

### Experimental Animals

Sixty-four female Sprague-Dawley rats (250 g body weight) were organized into 8 groups (8 animals per group). The control group consisted of rats without RLN injury, and in the other 7 groups, rats underwent surgical transection and reanastomosis of the right RLN. Animals were euthanized at 1, 3, 7, 14, 21, 28, and 56 days postinjury (DPI), respectively. Intrinsic laryngeal muscles from 4 animals per group underwent mRNA extraction and qRT-PCR. Larynxes from the remaining 4 animals per group were used in immunohistochemistry experiments.

For survival surgeries, animals were anesthetized using 75 mg/kg ketamine with 5 mg/kg xylazine via intraperitoneal injection. Postoperative analgesia was achieved using 5 mg/kg meloxicam via subcutaneous injection prior to

awakening. For nonsurvival surgeries, animals were euthanized using 100 mg/kg ketamine with 10 mg/kg xylazine. This 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 Columbia University Medical Center 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 scissors at the level of the seventh tracheal ring. The distal and proximal stumps were aligned with a gap <1 mm and laid back down within the tracheoesophageal groove. The ipsilateral superior laryngeal nerve (SLN) was identified and transected close to the larynx. Both ends were ligated and separated to avoid collateral innervation to the denervated intrinsic laryngeal muscles.<sup>31</sup> The surgical window was sutured closed. A 0° 4-mm endoscope (Karl Storz, Tuttingen, Germany) was inserted transorally to confirm right vocal fold paralysis. At the time of sacrifice, rats underwent another endoscopy to evaluate for continued paralysis, regained function, or synkinesis of the ipsilateral vocal fold.

### Tissue Preparation

At the designated survival period per group, animals were euthanized with intraperitoneal ketamine/xylazine as described above. The right posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA) muscles were isolated, dissected out, put in sterilized 0.5-mL tubes, frozen in dry ice, and then kept at -80°C.

To ensure the expression of LAMA1, animals were euthanized as described above and underwent transcatheter perfusion with 0.1 M phosphate buffer saline (PBS) (200 mL), followed by 250 mL 4% paraformaldehyde in PBS. The larynxes were removed and fixed for 2 hours with 4% paraformaldehyde. They were then immersed in 15% sucrose in PBS overnight and transferred to 30% sucrose in PBS, where they were kept until frozen sectioning in a cryostat. Larynxes were sectioned at a thickness of 16 µm per slice. The sections were stored at -80°C on glass slides coated in gelatin.

### qRT-PCR

RNA from the muscles was isolated using the Kimble Biomasher II tubes and pestles with QuickRNA Microprep kits, following the manufacturers' instructions (Zymo Research, Irvine, California).

Complementary DNA (cDNA) was generated from 50 ng total RNA in a thermocycler PCR System 2400 (Perkin Elmer, Waltham, Massachusetts) using iScript Reverse

**Table 1.** TaqMan Probes Used for Quantitative Reverse Transcription Polymerase Chain Reaction.

Rat Genes	Reference
GAPDH	Rn01775763
GDNF	Rn00569510
Netrin 1	Rn00586934
LAMA1	Rn01418829

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDNF, glial-derived neurotrophic factor; LAMA1, laminin 111.

Transcription Supermix (Bio-Rad, Hercules, California). To increase ultimate PCR yield, cDNA was then preamplified in the thermocycler using TaqMan PreAmp Master Mix (Thermo Fisher, Waltham, Massachusetts) and TaqMan Gene Expression Assays (primers) for our molecular targets: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GDNF, netrin 1 (NTN-1), and LAMA1, following manufacturer's instructions (**Table 1**). In attempt to improve LAMA1 mRNA detection, we increased the amount of mRNA used prior to cDNA synthesis to 0.1  $\mu$ g. However, this did not improve yield. cDNA amplification was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Waltham, Massachusetts). The relative expression of the internal control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the studied genes (GDNF, NTN-1, and LAMA1) were measured using TaqMan Gene Expression Master Mix FAST (Thermo Fisher). Cycling times and temperatures were consistent with the manufacturer instructions of the TaqMan FAST reagents. The relative quantification of gene expression was calculated using the comparative  $C_T$  method ( $2^{-\Delta\Delta C_T}$ ), where  $\Delta\Delta C_T$  represents the differences between the studied genes (GDNF, NTN-1, LAMA1) and the internal control gene (GAPDH).

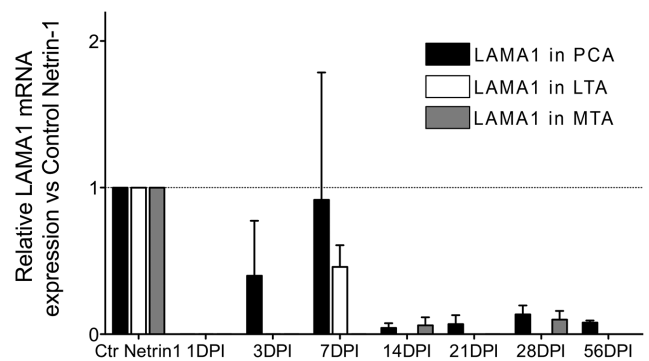
### Statistical Analysis

All numerical data obtained in the experiments were expressed as mean  $\pm$  standard error. Quantification of mRNA was analyzed by 1-way analysis of variance (ANOVA) and unpaired 2-tailed Student *t* test using Prism 5 software (GraphPad Software, La Jolla, California). Differences with a *P* value of less than .05 were considered statistically significant.

### Immunohistochemistry: Larynx

After euthanasia, rats were perfused transcardially with PBS, followed by 4% paraformaldehyde. Isolated larynxes were postfixed in 4% paraformaldehyde for 2 hours and then cryoprotected in 30% sucrose solution (15% sucrose in PBS for 1 hour, then 30% sucrose overnight). Sections were washed in Tris-buffered saline (TBS) and blocked in 5% BSA in TBS for 30 minutes at room temperature. Sections were incubated in 0.8% BSA in phosphate-buffered saline with .3% Triton (PBST) overnight at 4°C with 1:300 rabbit

## Expression of LAMA 1



**Figure 1.** Messenger RNA (mRNA) expression of laminin 111 (LAMA1) compared to control netrin 1 in posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA). DPI, days postinjury.

antilaminin antibody (ab11575; Abcam, Cambridge, Massachusetts). Slides were then incubated in goat anti-rabbit IgG Alexa Fluor 594 conjugate (1:200; EMD Millipore, Burlington, Massachusetts). To visualize motor endplates, the sections were then incubated in Alexa Fluor 488-conjugated  $\alpha$ -bungarotoxin (1:500) in TBS for 2 hours at room temperature. Coverslips were mounted with Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; blue) (Abcam) and stored at 4°C. Sections were evaluated using a Zeiss Axioskop epifluorescence microscope (Zeiss, Oberkochen, Germany).

## Results

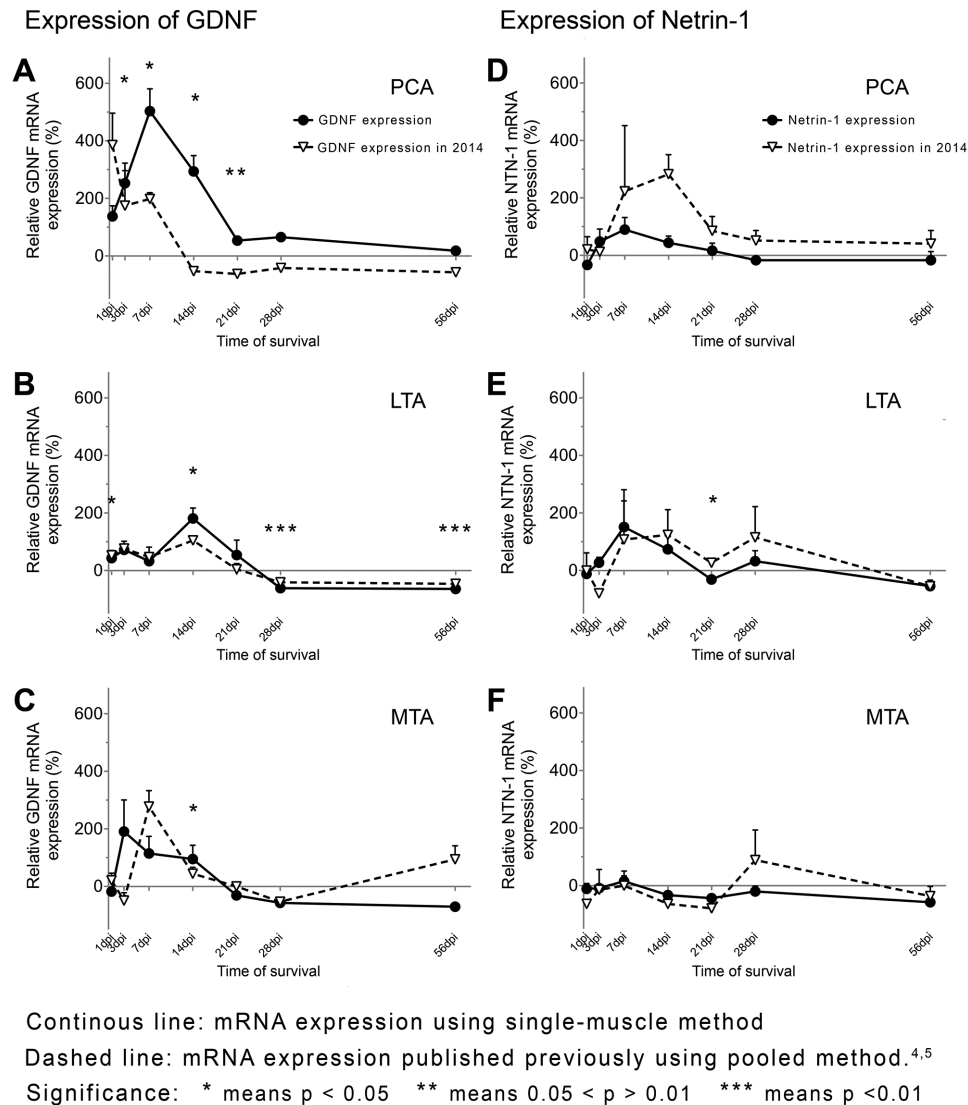
### Vocal Fold Motion Evaluation

In all experimental rats, normal vocal fold movement of the right vocal fold was observed immediately prior to right RLN transection and anastomosis, and no right vocal fold movement was observed immediately after. At the designated time points, the right laryngeal folds remained paralyzed except 2 rats at 28 DPI and 1 at 56 DPI, which exhibited disorganized, synkinetic movement of the right vocal fold.

### qRT-PCR

LAMA1 mRNA expression was not detected in control PCA, LTA, and MTA samples, indicating expression levels below the undetectable limit of the qRT-PCR machine. In the PCA, LAMA1 mRNA expression was present at low levels from 3 to 56 DPI. In the LTA, LAMA1 expression was detected at 7 DPI and in the MTA at 14 and 28 DPI (**Figure 1**).

Quantification of GDNF mRNA expression from PCA, LTA, and MTA muscles showed time-dependent changes in GDNF expression following right RLN injury. From 1 to 21 DPI, right PCA and LTA had higher GDNF expression levels than their left counterparts. In the right PCA, peak expression was observed at 7 DPI ( $P = .018$ ), followed by a



**Figure 2.** Messenger RNA (mRNA) expression of glial-derived neurotrophic factor (GDNF) (A-C) and netrin I (D-F) from 1 to 56 days postinjury (DPI) in the posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), and medial thyroarytenoid (MTA). Continuous line: single-muscle method. Dashed line: pooled method.<sup>4,5</sup>

dramatic drop below control levels. GDNF mRNA expression returned to control levels at 56 DPI. In contrast, in the LTA, peak GDNF mRNA levels were observed at 14 DPI ( $P = .047$ ), followed by a return to control levels. In addition, at 28 and 56 DPI in the LTA, GDNF mRNA expression was downregulated compared to control ( $P = .001$  at 28 and 56 DPI). GDNF mRNA expression in the MTA showed nonsignificant variation postinjury. These findings are consistent with our previous study using a different mRNA isolation technique to quantify GDNF expression, as shown in **Figure 2**.<sup>4</sup>

Netrin 1 mRNA expression displayed temporal variation in the PCA and LTA muscles of experimental group animals. At 1 DPI, netrin 1 expression remained at basal levels in the PCA, LTA, and MTA. mRNA expression increased slightly at 3 DPI in the PCA and LTA, reaching peak levels at 7 DPI and then decreasing to basal levels by 21 DPI in

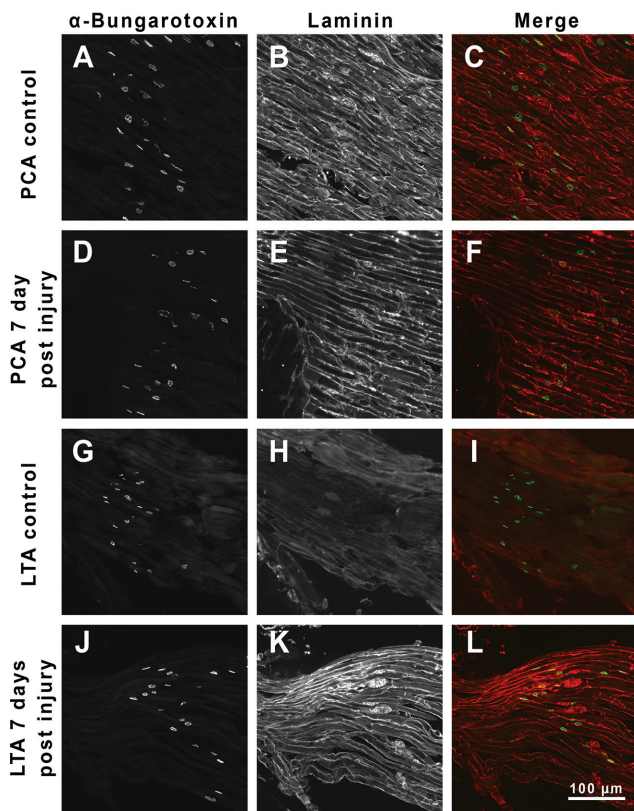
both muscles ( $P = .037$ ). MTA mRNA expression of netrin 1 did not vary significantly from control levels postinjury. Here we are also able to reproduce prior results using a different technique, as shown in **Figure 2**.<sup>5</sup>

### Immunohistochemistry

To confirm our qRT-PCR findings for LAMA1, we evaluated laminin protein expression via immunohistochemistry (IHC) of the laryngeal muscles at different time points. The trend seen in IHC corroborates the qRT-PCR findings, with a clear, positive immunoreaction for laminin at 7 DPI in the right LTA (**Figure 3**).

### Technical Considerations

The present study uses what we found to be an improved protocol for mRNA purification. Key modifications were as



**Figure 3.** Motor endplates (A, D, G, J) and laminin (B, E, H, K). Posterior cricoarytenoid (PCA) control (A-C) and PCA 7 days postinjury (DPI) (D-F) showed similar laminin III (LAMA1) staining. In contrast, clear differences were observed between lateral thyroarytenoid (LTA) control (G-I) and 7 DPI (J-L).

follows: first, tissue was disaggregated using Kimble Biomasher II tubes and pestles in buffer provided with the kit. Second, we performed brief sonication of the samples using a TissueRuptor from Qiagen (Qiagen, Hilden, Germany). In addition, we used the minimum recommended volume of buffer to maximize the concentration of components isolated from the tissue samples. The remaining steps of mRNA isolation were performed according to the manufacturer instructions. mRNA concentration measurement and PCR analyses were performed as commonly used for these procedures. With these modifications and the addition of the preamplification step using TaqMan PreAmp Master Mix (Thermo Fisher) and TaqMan Gene Expression Assays (primers), the samples generally exceeded the readable baseline limit of the PCR machine. All testing and experimental samples were read in triplicate, and measurements were consistent, indicating robust results following preamplification.

## Discussion

In the present study, we describe for the first time the expression of LAMA1 mRNA following RLN injury. LAMA1, a component of the extracellular matrix of muscles, may be a key player in axonal pathfinding during reinnervation.<sup>32,33</sup> In vitro experiments have shown that the combination of netrin

1 receptor deleted in colorectal cancer (DCC) expression in the axonal growth cone and LAMA1 expression in the muscle induces axonal repulsion in the presence of netrin 1.<sup>21</sup>

This study is the first to describe LAMA1 mRNA expression in laryngeal muscles following RLN injury. We showed that LAMA1 mRNA expression is upregulated following RLN transection and reanastomosis at different time points in the PCA, LTA, and MTA. Specifically, there is consistent, low-level LAMA1 mRNA expression in the PCA and narrow expression in the LTA and MTA at 7 and 14 DPI, respectively. In the LTA, we find that LAMA1 mRNA expression correlates with LAMA1 protein production. However, this was not the case in the PCA. One explanation is that the LTA and PCA differ in their regulation of LAMA1 translation postinjury. Another possibility is to consider is a limitation of qRT-PCR: LAMA1 expression sometimes falls below the detectable limit of the qRT-PCR machine, and thus it was not possible to quantify LAMA1 expression at all time points.

In addition, in this study, we are able to reproduce the GDNF and netrin 1 mRNA expression results from our previous studies.<sup>4</sup> Temporal expression patterns of these trophic factors are distinct in the PCA, LTA, and MTA, respectively. They provide insight into the molecular mechanisms underlying the sequential reinnervation that is observed after RLN injury, in which the PCA is reinnervated first at ~7 DPI, followed by the LTA and finally the MTA.<sup>4,5</sup> With these and prior data, we see that GDNF mRNA levels in the PCA peak at 7 DPI, correlating with the time at which the first reinnervating axons reach the PCA.<sup>4,34</sup> Based on our present observations, we could further postulate that following reinnervation of the PCA, GDNF expression is downregulated, allowing for maturation of the newly formed synapses. As GDNF expression wanes in the PCA, it peaks in the LTA at 14 DPI, promoting formation of neuromuscular synapses on the LTA and leaving the remaining axons to reinnervate the MTA.

Netrin 1 expression patterns suggest a more complex mechanism. First, we found greater internal variation in netrin 1 mRNA expression among samples of a given time point, compared to that of GDNF. Second, netrin 1 expression was found to peak at 7 DPI in the PCA, which correlates to the arrival of new axons to the PCA. However, peak expression in the LTA was also observed at 7 DPI, when axons have yet to reach the LTA. This discrepancy may be due to the many modulators of netrin 1 signaling. As noted, netrin 1 exhibits both attractant and repellent properties on the projection of motor axons.<sup>13,18,19</sup> This dual property is mediated through 2 receptors: DCC and UNC5. DCC can induce either attraction or repulsion of growing axons while UNC5 induces only repulsion.<sup>18,20</sup> Furthermore, LAMA1 is known to activate netrin 1 as a repellent signal. In this experiment, LAMA1 peaked in the LTA at 7 DPI and in MTA at 14 DPI, possibly resulting in axon repulsion, delaying LTA and MTA reinnervation. This correlates with the known timing and pattern of laryngeal reinnervation.<sup>4</sup>

Taken together, the molecular findings of this study advance the current knowledge of RLN regeneration by describing the spatial and temporal expression of LAMA1 in the context of netrin 1 and GDNF expression following RLN injury. However, how exactly these neurotrophic factors, their receptors, and LAMA1 cooperate in time and space to affect axon guidance requires further study. A therapeutic application of this research could be that manipulation of netrin 1 and LAMA1 expression at opportune time points could repel misguided axons from innervating the wrong muscle, enhancing selective reinnervation and reducing synkinesis.

Another novel aspect of this work is the application of a new protocol that optimizes mRNA purification from rat laryngeal muscles. Prior mRNA studies of rat laryngeal muscle have used the TRIzol method.<sup>4,5,27-30</sup> A major limitation of using this method in the rat model is the size of rat larynges. Investigators have previously pooled 2 to 6 muscles from different animals to obtain enough mRNA for analysis. Prior to the present study, only van Daele<sup>35</sup> in 2010 had proposed a method for robust gene expression quantification using a single rat laryngeal muscle. Despite van Daele's efforts, pooling has remained common practice in this field.

The protocol used here allowed us to obtain sufficient mRNA for qRT-PCR analysis using a single PCA, LTA, or MTA muscle. This, in turn, reduced the number of animals required for the study. It also improved our ability to examine the internal variation of mRNA expression between single animals. We attribute protocol improvement to 2 main factors: (1) improved disaggregation of tissue and (2) a preamplification step.

Suboptimal disaggregation of the laryngeal muscles contributes to suboptimal extraction of mRNA from a sample. Our 2-step process proved critical. We performed an initial disaggregation using a microfuge tube and pestle kit. We then used moderate sonication to further disaggregate samples. Samples that underwent moderate sonication showed higher concentrations of mRNA compared to those that had not.

Another critical step is preamplification of the samples. To our knowledge, despite its common use in other fields,<sup>36,37</sup> preamplification has not been used in the study of laryngeal muscles. To check the robustness of this new protocol, we compared the GDNF and netrin 1 mRNA expression results obtained in the present experiments to those from our prior studies using the pooling method.<sup>4,5</sup> With the exception of GDNF expression in the MTA, results were consistent between the present study and those in our previous works.

## Conclusion

LAMA1 expression exhibits temporal variation following RLN injury and anastomosis, especially in the LTA and MTA. This expression is correlated to the upregulation of netrin 1 and the timing of laryngeal muscle reinnervation. This finding and the known interaction between LAMA1

and netrin 1 in other systems suggest that LAMA1 plays a role in netrin 1 signal modulation in laryngeal muscle reinnervation. In addition, a new tissue-conserving method allows for mRNA isolation from single animal laryngeal muscles that will produce robust qRT-PCR results. Further investigation of netrin 1 and LAMA1 interaction during laryngeal reinnervation is warranted, and these new techniques will enhance future research.

## Author Contributions

**Michael B. Montalbano**, assisted in data acquisition through tissue processing, assisted in data analysis and interpretation through PCR, cowrote and revised manuscript; **Ignacio Hernández-Morato**, co-conceived the project, assisted in data acquisition through tissue processing, assisted in data analysis and interpretation through PCR, cowrote and revised manuscript; **Likun Tian**, assisted in data acquisition through tissue processing, assisted in revising manuscript; **Victoria X. Yu**, assisted in data acquisition through tissue processing, assisted in revising manuscript; **Sonam Dodhia**, assisted in data acquisition through tissue processing, assisted in revising manuscript; **Jose Martinez**, assisted in data analysis and interpretation through PCR, assisted in revising manuscript; **Michael J. Pitman**, co-conceived the project, cowrote and revised manuscript.

## Disclosures

**Competing interests:** None.

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