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Research article

Influence of Netrin-1 on reinnervation of laryngeal muscles following recurrent laryngeal nerve injury☆

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HIGHLIGHTS

- Recurrent laryngeal nerve injury leads to non-specific reinnervation of the larynx.
- Netrin-1 plays an important role during laryngeal muscle reinnervation.
- Injection of Netrin-1 reduces reinnervation of the posterior cricoarytenoid muscle.
- Netrin-1 injection alters the motor reinnervation process of the larynx.

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ABSTRACT

Following recurrent laryngeal nerve (RLN) injury, recovery results in poor functional restitution of the paralyzed vocal fold. Netrin-1 has been found to be upregulated in the rat posterior cricoarytenoid muscle (PCA) during nerve regeneration. We evaluated the effect of ectopic Netrin-1 in the PCA during RLN reinnervation. The right RLN was transected and Netrin-1 was injected into the PCA (2.5, 5, 10, 15, 20 μg/ml). At 7 days post injury fluorescent retrograde tracer was injected into the PCA and Thyroarytenoid (TA) muscles. At 9 days tissues were harvested. Immunostaining showed reinnervation patterns in the laryngeal muscles and labelled motoneurons in the nucleus ambiguus. Lower concentrations of Netrin-1 (2.5 and 5 μg/ml) showed no significant changes in laryngeal muscles reinnervation. Higher concentrations of Netrin-1 significantly reduced motor end plate innervation. The most effective dose was 10 μg/ml showing reduced number of innervated motor endplates in the PCA. The somatotopic organization of the nucleus ambiguus was altered in all concentrations of Netrin-1 injection. These findings indicate that injection of Netrin-1 into the PCA changes the reinnervation pattern of the RLN.

1. Introduction

Surgical procedures of the neck and upper chest may result in injury of one or more peripheral nerves including the recurrent laryngeal nerve (RLN). The RLN supplies all intrinsic muscles of the ipsilateral vocal fold except the cricothyroid muscle, which is supplied by the superior laryngeal nerve (SLN). Injury to the RLN can result in vocal fold paresis or paralysis that leads to loss of normal voice, airway obstruction, and possible aspiration [1]. After nerve transection reinnervation of the larynx is non-selective and vocal fold motion is never restored to baseline [1–3].

A number of growth factors have been shown to participate in the guidance of regenerating axons following nerve injury [4,5]. In the rat, transection of the RLN leads to ipsilateral vocal fold paralysis and denervation of the intrinsic laryngeal muscles [4,6–9] followed by upregulation of trophic factors, such as NGF, GDNF, and Netrin-1, within the muscles [5,10,11]. However, axons are not appropriately guided back to their original muscles and non-selective reinnervation of the larynx occurs resulting in synkinetic movements [4,9,10].

Netrin-1 is a member of the extracellular laminin-related protein family which is phylogenetically conserved across many species [12]. In the central nervous system (CNS), Netrin-1 is expressed in the floor plate of the neural tube during development [13–17] and is critical for axonal projections that cross the

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midline in the spinal cord, brainstem, and other regions of the brain [15,16,18–20]. Netrin-1 has also been shown to participate in the guidance of retinal axons [19,21] and sensory projections from spiral ganglion cells during inner ear development [22,23]. Specifically, when Netrin-1 is expressed in the developing trochlear nucleus, axons are repelled from the floor plate and projected dorsally to cross the midline [16,20]. While Netrin-1 plays a chemorepulsive role close to the floor plate, it also acts as a chemoattractant near the exit points of developing axons within the brainstem [17,24].

In adults, following peripheral nerve injury, Netrin-1 is upregulated in the distal segment of the nerve and may serve an axonal pathfinding role during nerve regeneration [25,26]. However, the definitive role of Netrin-1 in denervated muscles is unclear. Earlier studies have shown that following RLN injury, the expression of Netrin-1 is upregulated in select intrinsic laryngeal muscles but not in others [11]. The aim of this study is to evaluate the effect of Netrin-1 in the posterior cricoarytenoid muscle (PCA) during RLN regeneration.

2. Material and methods

2.1. 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. Forty-two adult female Sprague Dawley rats (250–300 g) were distributed into seven groups (six animals per group). The first control group consisted of rats in which there was no RLN injury and no injection performed. In the second control group, the right RLN was transected and re-anastomosed and 0.1 M phosphate buffer saline (PBS, pH 7.1) was injected into the right PCA. In the experimental group (30 rats, six animals per Netrin-1 concentration), the right RLN was transected and re-anastomosed, and Netrin-1 (2.5, 5, 10, 15, 20 μg/ml) was injected into the right PCA at three days post injury (DPI).

2.2. Surgical protocol: RLN transection

Animals were 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 & Upjon, New York, NY) was placed beneath the RLN, which was then transected with an iridectomy scissor. The distal and proximal stumps were then aligned with a gap of less than one millimeter. The anastomosis was then secured by another piece of gelfoam on top of both stumps. The ipsilateral SLN was identified and transected close to the larynx; both ends were ligated and separated in order to avoid collateral innervation to the denervated intrinsic laryngeal muscles [27]. The surgical window was then sutured and animals were returned to their facility with supply of food and water “ad libitum”.

2.3. Surgical protocol: injection of Netrin-1

At 3 DPI the surgical window was reopened. In order to expose the PCA, the larynx was slightly rotated and the right inferior constrictor muscle was incised. A glass microelectrode with a 30 μm opening attached to a Hamilton Syringe (Hamilton Company, Reno, NV) was inserted into the PCA using a micromanipulator on a stereotaxic setup. Two microliters of the desired concentration of Netrin-1 (R&D Systems, Minneapolis) were then slowly injected into the PCA. A piece of gelfoam was then placed over the PCA injection site and the surgical window was closed. The injection was performed at three DPI with the rationale that at this time, axons are in close proximity to, but not yet reaching the larynx.

2.4. Injection of retrograde tracer into laryngeal muscles

A third surgery was performed at 7 DPI (four days after PCA injection) for neural tracer injection into the PCA and thyroarytenoid (TA) muscles. The larynx was exposed as described above, and 0.5 μl of Cholera Toxin subunit B (CtB) conjugated to Alexa Fluor 488 (CtB-AF488) (Millipore, Billerica, MA) was injected into the PCA [9,28]. For the TA muscle, 0.5 μl CtB conjugated to Alexa Fluor 594 (CtB-AF594) was injected via a window through the thyroid cartilage.

2.5. Functional evaluation

Vocal fold movement was assessed with a trans-orally inserted 0° 4 mm endoscope (Karl Storz, Germany). Movement was recorded by a camera attached to the laryngoscope with a digital recording system. Vocal fold movement was evaluated and recorded immediately after nerve transection and immediately before the animal was sacrificed at 9 DPI [29].

2.6. Immunohistochemistry

Animals were euthanized with isoflurane inhalation at 9 DPI. Animals were perfused transcardially with cold PBS followed by 4% paraformaldehyde. Isolated larynges and brainstems were post-fixed by immersion in the same fixative for 30 min, transferred to 15% sucrose in PBS overnight, and transferred to 30% sucrose for cryoprotection. Larynges were cut in 30 μm serial sections in a Leica cryostat.

Sectioned laryngeal muscles were incubated with rabbit anti-β-tubulin III polyclonal antibody (1:1000, Covence) in TBS with 0.8% BSA for 48 h at 4 °C. After washing, the sections were incubated in Cy3 conjugated anti-rabbit IgG (1:400, Jackson Lab) for 1 h at 4 °C. Sections were then incubated in AlexaFluor 488 conjugated α-bungarotoxin (1:500) for two hours at room temperature. Sections were mounted with glycerine–PBS 1:1 solution. The confocal microscopic images were used to evaluate innervation of the motor endplates.

All results are expressed as mean ± standard deviation. For comparison of the PCA and TA categories among experimental groups, two-way ANOVA tests were used to determine statistical significance. The level of significance was set at $p < 0.05$. The two-tailed unpaired Student’s *t*-test was used to compare reinnervation of the control and experimental groups per muscle following RLN injury.

50 μm serial coronal sections of the brainstem were analyzed using a Zeiss Axioskop microscope. The number and position of motoneurons innervating specific muscles were delineated in the nucleus ambiguus due to labelling by cholera toxin conjugated by two different fluorochromes.

3. Results

Controls and all experimental rats showed no vocal fold motion (grade 0) immediately after RLN injury and just before sacrifice at 9 DPI.

3.1. Immunohistochemistry – laryngeal muscle analysis

In control group 1, 100% of labelled motor endplates in the PCA, LTA and MTA were innervated (Figs. 1 and 2) and results were similar to previous studies [10]. In the PBS control group, 75% of PCA motor endplates were reinnervated, while the LTA and MTA showed 9% and 6% reinnervation, respectively. ANOVA testing showed significant differences between the PCA and TA reinnervation.

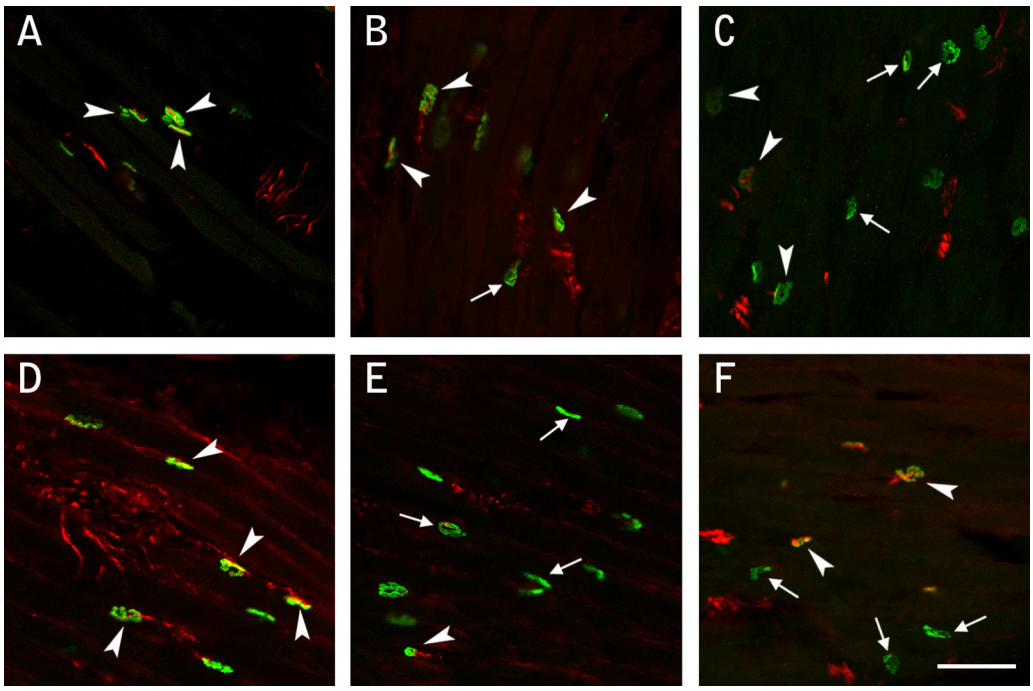

Fig. 1. Confocal images showing motor axons (red) and non-innervated motor end plates (MEPs) (green, white arrow) in the posterior cricoarytenoid (PCA) (A, B and C) and the lateral thyroarytenoid (LTA) (D, E and F) muscles. MEPs innervated by motor axons merged to form yellow (white arrowheads). (A and D) Normal innervation of MEPs in control group 1 before nerve injury. (B) MEP innervated in the PCA following nerve injury. (C) Less innervated MEP following nerve injury and injection of 10 μg/ml Netrin-1. (E) Nearly no MEP innervation following nerve injury and injection of 2.5 μg/ml Netrin-1. (F) Increased MEP innervation following nerve injury and injection of 10 μg/ml Netrin-1. The bar represents 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

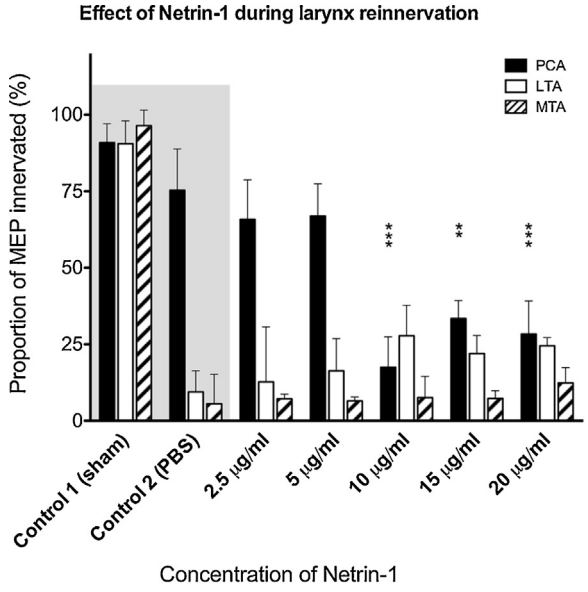

Fig. 2. Number of neuromuscular junctions formed following RLN injury in the PCA, LTA, and MTA at different Netrin-1 concentrations. In the PBS control group and lower concentration groups (2.5, 5 μg/ml), innervation in the PCA was more strongly identified compared to the LTA and MTA. In the higher concentration groups (10, 15 and 20 μg/ml), there was significantly decreased PCA innervation, with a trend towards increased LTA innervation compared to PBS controls. The number of MEPs in the MTA was constant across all groups except control 1. Error bars = standard deviation of mean; $^{**}0.01 < p < 0.05$; $^{***}p < 0.01$. PCA: posterior cricoarytenoid; LTA: lateral thyroarytenoid; MTA: medial thyroarytenoid; RLN: recurrent laryngeal nerve; MEP: motor end plate.

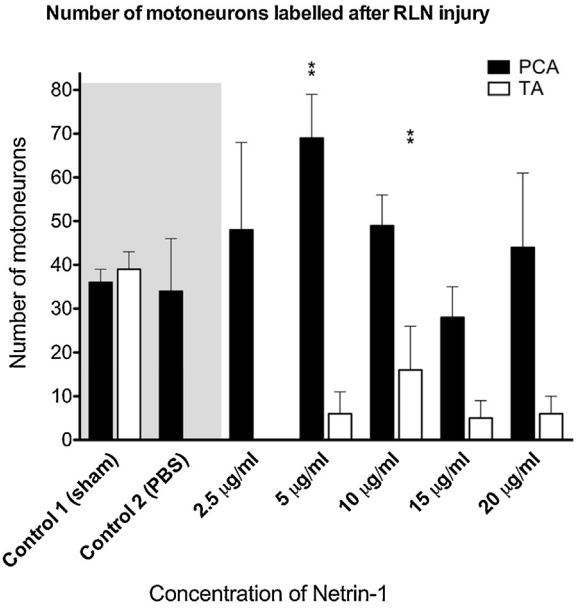

Fig. 3. Number of motoneurons labelled in the ipsilateral nucleus ambiguus after injection of CtB tracer into the PCA and TA muscles. Following RLN injury, more labelled motoneurons were traced in the PCA compared to the TA across all study groups. Compared to PBS controls, the number of labelled motoneurons in the PCA were significantly increased at 5 μg/ml and the number of labelled motoneurons in the TA were significantly increased at 10 μg/ml. Error bars: standard deviation of mean; $^{**}0.01 < p < 0.05$; CtB: subunit B of Cholera Toxin tracer; PCA: posterior cricoarytenoid; TA: thyroarytenoid; RLN: recurrent laryngeal nerve; MEP: motor end plates.

Differences in motor innervation were found in animals with different concentrations of Netrin-1 injected. Rats treated with 2.5 and 5 μg/ml of Netrin-1 showed motor end plate innervation that was similar to the PBS control group (Fig. 2). Specifically, these groups demonstrated slightly less reinnervation in the PCA (75% in PBS controls, 66% with 2.5 μg/ml, and 67% with 5 μg/ml), and slightly more innervated motor endplates in the LTA (9% in PBS controls, 11% with 2.5 μg/ml, and 16% with 5 μg/ml).

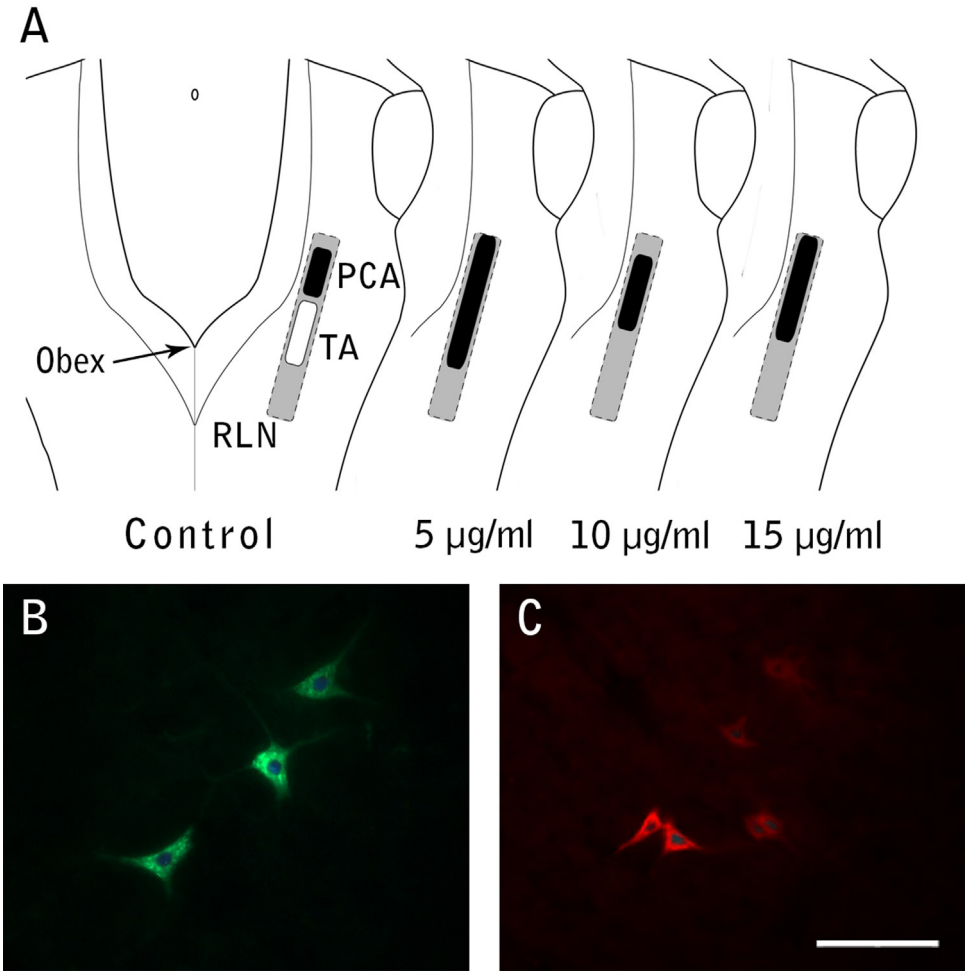

Fig. 4. Position of motoneurons within the nucleus ambiguus following injury of the recurrent laryngeal nerve and injection of Netrin-1. (A) Diagram summarizing the position of motoneurons innervating PCA and TA muscles before injury, and after nerve injury and injection of different concentrations of Netrin-1. (B) Neurons labelled from control PCA in green. (C) Neurons labelled from control TA in red. PCA: posterior cricoarytenoid; TA: thyroarytenoid; RLN: recurrent laryngeal nerve; MEP: motor end plates. The bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

After injection of 10 μg/ml of Netrin-1, compared to the PBS control group, the number of innervated motor endplates decreased significantly in the PCA (17%, $p \leq 0.001$) and increased in the LTA (27%, $p \leq 0.002$). In the groups with 15 and 20 μg/ml concentration the number of innervated plates in the PCA was increased by 28% in both groups, as compared to the group with 10 μg/ml Netrin-1 (17%). But, this number substantially decreased compared to the group with 2.5 or 5 μg/ml (66 and 67%, respectively). The number of labelled motor endplates in the LTA was similar between the 10, 15, and 20 μg/ml Netrin-1 groups, which were all increased, compared to the PBS control (Fig. 2). In the MTA, there were no differences between the PBS control group and any of the experimental groups (Fig. 2).

3.2. Immunohistochemistry – brainstem analysis

The number of labelled motoneurons in the nucleus ambiguus in the normal control group was 36 ± 3 for the PCA, and 39 ± 4 for the TA which was similar in number to previous studies [28]. The number of labelled motoneurons in the PBS control group (rats with transaction of RLN plus application of the tracer) was 34 ± 13 in the PCA, with no labelled motoneuron in the TA (Fig. 3). Differences between the PCA and the TA categories were observed according to ANOVA analyses.

In the experimental groups, mean number of labelled neurons from the PCA ranged from 28 to 69. The number of labelled motoneurons in the 2.5 μg/ml Netrin-1 group was 47 ± 20. This number increased significantly in the 5 μg/ml group to 69 ± 10 ($p < 0.001$). The number of labelled neurons in the 10 and 20 μg/ml groups were 49 ± 7 and 44 ± 17, respectively.

There was no labelled motoneuron observed from the TA after Netrin-1 injections of 2.5 and 5 μg/ml. In the 10 μg/ml Netrin-1 group, 16 ± 10 labelled motoneurons were observed, whereas very few labelled neurons (5 ± 4 and 6 ± 4 respectively) were observed in 15 and 20 μg/ml groups.

Motoneurons were identified at different locations in the nucleus ambiguus depending on the experimental intervention. In control group 1, labelled motoneurons to the PCA were identified in the rostral nucleus ambiguus, and TA motoneurons were located caudal within the nucleus. In contrast, the PBS control group and experimental groups demonstrated labelled motoneurons were distributed along the length of the ipsilateral nucleus ambiguus in a disorganized somatotopic arrangement. There were no significant differences regarding the position of labelled motoneurons in the differing experimental concentration groups (Fig. 4).

4. Discussion

Results from both control groups in the present study confirm previous findings regarding both the timing and patterning of reinnervation following RLN injury and re-anastomosis, as well as the location of brainstem motoneurons post-innervation [4,10]. Results

showed that regenerating axons first reinnervate the PCA muscle at 7 DPI, followed by delayed innervation of LTA and MTA. It further showed that the location of PCA motoneurons at the earliest stages of reinnervation were demonstrated at their expected location. In addition, a few neurons labelled in the nucleus ambiguus were shown to be in the unexpected territory of the TA motoneurons [9,28,30–34].

Experimental animals showed that lower concentrations of Netrin-1 (2.5 and 5 μg/ml) injected into the PCA allowed for axons to innervate more robustly when compared to higher concentration of Netrin-1 (10, 15 and 20 μg/ml). In previous studies, it has been reported that regenerating axons reached the PCA at 7 DPI and the LTA at 14 DPI [4,10]. In the present study we observed an alteration in the PCA reinnervation at 9 DPI.

A concentration of 10 μg/ml had the most robust impact for retarding the innervation of axons into the PCA and demonstrating a trend towards increased reinnervation of the LTA. This however correlated to an increase, rather than decrease in the number of labelled brainstem motoneurons compared to controls. The reason for such discrepancy is unclear, but these observation are consistent with prior studies evaluating early stages of reinnervation [9,34].

Netrin-1 has been shown to play several roles during central and peripheral nervous system development [12]. Early description of Netrin-1 involvement in the formation of the neural tube delineates its role in the repulsion of axons from the floor plate [13,15] and projection of commissural axons [16,18]. Netrin-1 has both attractant and repellent properties that have been described in axonal projections in the developing brainstem [14,17,20,22,23,35–37]. This guidance of motor axons is manifested through the binding to two different Netrin-1 receptors that modulate signal transduction to the cell body [38,39]. The DCC receptor modulates both attraction and repulsion of growing axons while the UNC5 receptors induce only chemorepulsion [24,36].

Relatively less is known about the role of Netrin-1 during nerve regeneration, though an upregulation of Netrin-1 by Schwann cells has been observed in degenerated nerve segments following acute injury, suggesting an attractive role of Netrin-1 during nerve regeneration [25,26]. Prior works have also demonstrated an association between the timing of changes in Netrin-1 expression following RLN injury and reinnervation of PCA and LTA muscles, but not in the MTA [11]. Other studies have demonstrated similar upregulation of GDNF in the PCA, LTA, and MTA following nerve injury. This difference between Netrin-1 and GDNF expression in the MTA may play an important role in the patterning of reinnervation as the MTA is the last muscle to be innervated. Downregulation of GDNF expression during reinnervation promotes the maturation of neuromuscular synapses [4,10]. The possibility of a cooperative role of these factors with the timing of the expression of GDNF and Netrin-1 receptors in the axonal growth cone during the reinnervation process require further investigation.

The present study showed that higher concentrations of Netrin-1 reduced reinnervation of the PCA and may induce reinnervation of the LTA. The altered somatotopic map of the nucleus ambiguus suggests the non-selective reinnervation. Further study of selection and maturation of neuromuscular junctions during RLN reinnervation after Netrin-1 injection, as well as Netrin-1 receptor expression during nerve regeneration, will enhance our understanding of the role of Netrin-1 during laryngeal muscle reinnervation.

5. Conclusion

In the present study, through application of different concentrations of Netrin-1 in the PCA, it was demonstrated that the presence of higher concentration of Netrin-1 lower the number of motor end plates innervated in the PCA. Because the innervation of regenerated axons is temporaly regulated, the first wave of regenerating axons innervate the PCA containing both the abductor and adductor motoneurons. This pattern leads to a breakdown of normal somatotopic representation resulting in synkinesis. Since ectopic innervation in the PCA is never corrected, the resulting innervation to the LTA and MTA is reduced.

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Conflict of interest

No authors have any disclosures.

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