Original Article

Neurotrophin expression and laryngeal muscle pathophysiology in response to recurrent laryngeal nerve-crush injury

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Abstract: Laryngeal palsy often results from recurrent laryngeal or vagal nerve injury during oncologic surgery of the head and neck. The aim of this study was to describe the characters of spontaneous reinnervation in rats following recurrent laryngeal nerve (RLN) crush injury. Twenty-five male Sprague-Dawley rats each were subjected to a 5-mm-long crush injury to the right recurrent laryngeal nerve. Vocalization, vocal fold movements, histological factors (e.g., axonal diameters, axon numbers), and immunostaining measures of neurotrophin activity were recorded at different time points (i.e., 3 weeks, 6 weeks, 10 weeks, and 16 weeks) following injury. Results showed that vocalization and vocal fold movement were restored at week 16. The expression of brain-derived neurotrophic factor (BDNF) and glial-cell-line-derived neurotrophic factor (GDNF) was different in thyroarytenoid (TA) and posterior cricoarytenoid (PCA) muscles. The number of axons returned to baseline at week 16, whereas axon diameter was not statistically significantly different between the injured and control axons at week 10. During regeneration of RLN, differences in the expression of neurotrophic factors may lead to the preferential reinnervation of TA. The study shows that application of neurotrophic factors could help restore recurrent laryngeal nerve function.

Keywords: Recurrent laryngeal nerve, nerve regeneration, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factors, rats, sprague-dawley

Introduction

Most functions of the larynx depend on healthy vocal fold motion, which, in turn, relies on an intact recurrent laryngeal nerve (RLN) [1]. The cause of injury (e.g., viral infection, trauma, invasion by malignant tumors) results in different degrees of damage [2] and different symptoms and signs of laryngeal paralysis [3]. Although some patients with unilateral vocal fold paralysis are completely asymptomatic, others experience disabling symptoms (e.g., serious hoarseness, dysphagia, dysphonia) [4]. Further, the effects of these symptoms could be devastating and impose psychosocial and economic burdens [5].

Although the injured RLN has a tendency to regenerate, even across a gap [6, 7], recovery of normal function is not absolute, due to laryngeal muscle atrophy, motoneuron loss, decrease in motor fiber density, or “synkinesis” [8-11]. Further, the potential regeneration of RLN depends on the mode and extent of injury [12].

Neurotrophic factors are a group of proteins that have been shown to prevent motoneuron loss and promote cell differentiation and reinnervation [13-18]. On the basis of prior research, we selected two kinds of neurotrophic factors, BDNF and GDNF, to study their expression after nerve injury.

Although spontaneous reinnervation is a common phenomenon after RLN injury and has been examined in a series of studies, vocal fold movement recovery takes a long time and
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is unpredictable and incomplete [19-23]. These studies mostly focused on the differences among neurotrophic factors following different nerve or muscle injuries. We established a model system using semi-quantitative and qualitative analysis to study phonatory recovery and evaluate the spontaneous reinnervation after RLN incomplete injury.

In our study, we chose rats as experimental animals for RLN injury research. Although the rat larynx is small, it has been used successfully for studying RLN injury and recovery [24]. In such previous studies, the peripheral nerve injury models focused on transecting [25-27], compressing [28, 29], stretching [30, 31], crushing [32-34], cutting [35, 36] and cauterizing [37, 38]. We did our research using a rat RLN crushing model.

Materials and methods

Experimental animals

Approval was obtained from the Animal Experimental Committee of the First People’s Hospital. Twenty-five male Sprague-Dawley rats weighing 320-350 g were maintained in the animal laboratory, and all surgical procedures were performed in the animal operating room. Treatment of the animals was in accordance with ethical and humane procedures.

Surgical procedure

Following intraperitoneal anesthesia with sodium pentobarbital (40 mg/kg body weight), telescopic video laryngoscopy was performed to confirm normal motion of the vocal fold with breathing. The right RLN was visualized along the tracheoesophageal groove. Rats were assigned randomly into two groups. For the denervation group, we produced a 5-mm-long crush injury at the level of the 7th tracheal ring by applying a steady force for 60 s twice to ensure a full crush with an aneurysm clip [39, 40]. For the control group, the procedure was identical to the one for the crush surgery except that the incision was sutured after the right recurrent laryngeal nerve was identified. Telescopic video laryngoscopy was repeated to confirm paralysis of the right vocal fold. If the right vocal fold displayed any movement, the rat was excluded from further study.

Measurement of vocalization

The animals were placed in a quiet environment the day before the procedure to measure vocalization. We recorded their vocalizations for 20 s during irritating stimulation of their right hind limb at 4-hr intervals following surgery. The procedures were performed in an identical environment by the same investigator using the same equipment, crush pressure, and distance between the recorder and mouth. Then, we used an audio capture tool (Cool Edit Pro v2.1) to intercept the sound fragments and then used Adobe Photoshop software to analyze the acoustic space.

Calculation of changes was performed as follows:

\[
\% \text{ Area} = \frac{\text{Postoperative area}}{\text{Area of control}}.
\]

\[
\% \text{ Amplitude} = \frac{\text{Postoperative amplitude}}{\text{Amplitude of control}}.
\]

Movement of vocal fold

Video laryngoscopy was performed as described above to assess vocal fold motion and glottis morphology during spontaneous breathing, at each of the following four postoperative time intervals: 3 weeks, 6 weeks, 10 weeks, and 16 weeks. The movements observed via transoral endoscopy were divided into 4 different categories, ranging from Grade 0 to Grade 3 [41]. The final score was obtained by two investigators who were blind as to the experimental group to which the animal had been assigned and blind as to the time since denervation. If the researchers’ ratings disagreed, a third investigator’s rating was obtained. Normal movement of the vocal fold was measured using five rats. Double-blind assay was performed in order to avoid subjective bias.

In addition, two frames of the vocal cord images were obtained to measure the arytenoid cartilages movement: one frame showed the fully abducted position and the other showed the fully adducted position [42]. The angles between arytenoid cartilages were calculated from the images of maximal adduction and maximal abduction (Figure 1A). Studies have shown that the effects of anesthetic on vocal fold mobility are minimal [43, 44].
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Histologic examination

After euthanasia, the larynx was excised. Under a dissecting microscope, the thyroarytenoid (TA) and posterior cricoarytenoid (PCA) muscles were removed to prepare them for immunohistochemistry staining for BDNF and GDNF. For each of the postoperative elapsed time interval, we obtained 6 slices of these muscles from each rat and prepared them for staining.

Nerve histology

The RLN was harvested from each animal, and the left nerve served as a control and was harvested at the same level. The RLN was isolated and fixed in 4% (vol/vol) formaldehyde for 48 h. Then we embedded the segments in paraffin, cut 7 μm sections from each segment, and examined them by Bielschowsky Modification. As a result, the axons appeared as black [45].

Measurement of immunostaining

Two observers, who were blind to the experimental conditions, examined each section separately using a morphometric workstation running Image Proplus 6.0 software. After calibrating the optical density, the images were recorded at a final magnification of ×100. Area, mean density, and relative optical density (ROD) of immunolabeling were obtained from each specimen. To make sure the ROD values were in the linear response range, we calibrated the microscope illumination.

Figure 1. The images and angles viewed by laryngoscopy. A: The images of control (Aa, Ab) and denervation groups at 3 (Ba, Bb), 6 (Ca, Cb), 10 (Da, Db) and 16 (Ea, Eb) weeks in order on maximal abduction (a) and maximal adduction (b) positions. B: The open angle at different times. The open angle of the denervated group increased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. C: The closed angle at different times. The closed angle of the denervated group decreased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. *P<0.05 vs. the control group.
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For each of the four postoperative elapsed time intervals, 6 histological sections of each rat’s nerve were prepared for photographic imaging. The diameter and volume of the axon were calculated with PS CC software, then the longest, shortest, and average diameters were determined.

**Table 1.** The assessment of vocal fold movement at different times

<table>
<thead>
<tr>
<th>Group</th>
<th>Control group</th>
<th>Third week</th>
<th>Sixth week</th>
<th>Tenth week</th>
<th>Sixteenth week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 2. The voice spectrums, area percentages and amplitude percentages at different times. (A) The voice spectrums of control (a) and denervation groups at third (b), sixth (c), tenth (d), sixteenth week (e). It reveals the amplitude and continuous wide waveform increased gradually. (B) The area percentage at different times. The spectrum area percentage of the denervated group increased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. (C) The amplitude percentage at different times. The amplitude percentage of the denervated group increased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. *P<0.05 vs. the control group.

**Statistical analysis**

The outcome value was summarized as a mean ± the standard deviation (SD). A one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test was used to perform the statistical analyses of the measures of vocalization and vocal fold movement using the Statistical Package for the Social Sciences (SPSS) version 20.0. The immunostaining and histological results were analyzed using the independent-samples T test. Differences between groups were considered to be significant at P<0.05.
Results

Animal procedures

All animals survived to the chosen endpoint. All incisions healed well, without infection. Nerve ends were surrounded with scar adhesions, with no evidence of significant swelling.

Vocal fold motion analysis

All denervated animals had right vocal fold paralysis. Detectable vocal cord movement was recorded, and the movement recovered by

Measurement of vocalization

The voice spectrums of denervated and control groups are shown in Figure 2A. Control rats emitted sharp and high vocalizations, while denervated rats emitted hoarse and deep vocalizations. The spectrum analysis of the control group showed high amplitudes with chronically wide waveforms (Figure 2Aa). After RLN injury, the spectrum was characterized by a narrower range of wavelengths and lower amplitudes. As time passed, the amplitude and frequency range of the vocalizations increased gradually (Figure 2Ab-Ae).

The spectrum area percentage of the denervated group increased gradually over time (Figure 2B). As can be seen from the chart, the area percentages of the corresponding four postoperative time points are 22.58±17.78, 32.45±3.91, 53.79±10.51, 98.41±8.04, respectively. The denervation groups’ vocalizations assessed at 3, 6, and 10 weeks showed a statistically significant difference (P<0.01) compared to the control group’s vocalizations. The postoperative amplitude percentages as determined at 3, 6, 10, and 16 weeks were 73.40±31.49, 87.23±9.04, 93.83±8.87, 100.00±10.85, respectively (Figure 2C). At 3, 6, and 10 weeks, amplitude percentages showed improvements (P<0.05), but there was no statistical difference between the control and denervated animals at 16 weeks (P = 1.00).

Figure 3. Analysis of immunohistochemistry in BDNF staining. A: Immunohistochemistry of right PCA (a), right TA (b), left PCA (c) and left TA (d), stained for BDNF for the control group, 3, 6, 10 and 16 weeks in sequence. Magnification, × 100. B: The expression of BDNF in PCA muscles. C: The expression of BDNF in TA muscles. *P<0.05 right TA muscle vs. the corresponding muscle of the control group. *P<0.05 left TA muscle vs. the corresponding muscle of the control group.
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week 16 following nerve crush (Table 1). A statistically significant difference was found for the angles between arytenoid cartilages for the denervation group (at 3, 6 and 10 weeks post-surgery) and the control groups (P<0.05). The angles of maximal abduction for the denervated group were 26.50±3.76, 31.27±4.22, 32.35±6.20 and 37.42±5.90 at 3, 6, 10, and 16 weeks, respectively, and 40.60±9.36 for the control group (Figure 1B). Moreover, the maximal adduction angles were 9.84±1.34, 6.90±0.92, 5.73±1.23, 4.65±0.64 and 4.51±1.05 for the four postsurgical time points and the control group, respectively (Figure 1C). By postsurgical week 16, the vocal fold movements at the site of the nerve-crush injury had returned to normal.

**Measurement of immunostaining**

Figure 3 graph the expression of BDNF in TA and PCA muscles. In the two denervated muscles, the expression of BDNF had significantly decreased by week 3 after injury. Although the expression quantity increased over time, it was markedly higher than the baseline level at week 6.

Expression of GDNF (Figure 4) in the right TA and PCA muscles had decreased by week 3 after surgery. Although only a negligible increase was observed in GDNF expression of the right TA from week 6 to week 10, it was higher than in the control group at week 6. The expression of GDNF in the right PCA was similar to variation tendency in the right TA. GDNF expression was higher of the left TA and PCA muscles in the denervation group than in the control groups at week 10 and 12.

**RLN histologic section analysis**

In some cases, there were compressed axons, and degenerating ones in others. Figure 5A shows axons embedded in a fibrous structure. Axons from the denervation group at 16 weeks showed no significant differences compared to those from the control group, about 121.50±6.61 axons (Figure 5B). The mean diameter of

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**Table 1**

<table>
<thead>
<tr>
<th>Time (week)</th>
<th>Angles between arytenoid cartilages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>26.50±3.76</td>
</tr>
<tr>
<td>6</td>
<td>31.27±4.22</td>
</tr>
<tr>
<td>10</td>
<td>32.35±6.20</td>
</tr>
<tr>
<td>16</td>
<td>37.42±5.90</td>
</tr>
</tbody>
</table>

**Figure 4.** Analysis of immunohistochemistry in GDNF staining. A: Immunohistochemistry of right PCA (a), right TA (b), left PCA (c) and left TA (d), stained for GDNF for the control group, 3, 6, 10 and 16 weeks in sequence. Magnification, × 100. B: The expression of GDNF in PCA muscles. C: The expression of GDNF in TA muscles. *P<0.05 right TA muscle vs. the corresponding muscle of the control group, *P<0.05 left TA muscle vs. the corresponding muscle of the control group.
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Figure 5. The schematic diagram, number and diameters of axon by Bielschowsky Modification. A: Red arrows indicate the axon. Scale bar, 20 μm. B: The number of axons at different times. The number of axons in the denervated group increased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. C: The average diameter of axons at different times. D: The maximum diameter of axons at different times. E: The minimum diameter of axons at different times. The minimum diameter of axons in the denervated group increased gradually over time and there was no statistical difference between the control and denervated animals at 16 weeks. *P<0.05 vs. the control group.

RLN axon sections was 4.38±1.75 μm (Figure 5C) in normal rats and increased over time among denervated animals at weeks 3 and 6. By postsurgical week 10, axonal diameter had returned to the baseline level in the latter animals, however.

Discussion

Nerve crush induces Sunderland second-degree injury [46], which involves injury to the axon [47]. It simulates the causes of RLN injury that are sustained in an intact nerve during idiopathic vocal fold paralysis or following iatrogenic traction injuries and during neurodegenerative diseases [48]. Because of ischemia, electrolyte concentrations shift and excitatory amino acids are released in excess [49], and these effects, in turn, initiate cytotoxic-signaling cascades, myelin destruction, and hamper the re-growth of axons [47]. From a clinical perspective, although crush injury does not represent the most severe neural injury, it can initiate injury that is consistent with axonotmesis, and, therefore, can serve as an ideal model to study the characteristics of spontaneous reinnervation of the nerve [50].

Intrinsic laryngeal muscles play an important role in phonation, respiration, and swallowing. They are innervated by two nerves: the RLN and the external branch of the superior laryngeal nerve (eSLN). The eSLN innervates the cricothyroid (CT) muscle, while the RLN terminates in the lateral cricoarytenoid (LCA), arytenoid (AR), thyroarytenoid (TA), and posterior cricoarytenoid (PCA) muscles [2]. Because the TA muscle is the most affected laryngeal muscle after injury of the RLN and PCA muscle is the exclusive abductor muscle, we chose these two muscles for our study [51]. The effects of RLN injury often present common clinical issues (e.g., dysphagia, dysphonia) including aspiration, in some cases [52]. Vocal folds are very delicate structures, and pretersensual injuries
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can result in excessive complications [47]. Injury to one or both nerves causes clinical problems and the prognosis for functional recovery is challenging [12].

In this study, vocalization, recovery of vocal fold movement, expression of BDNF and GDNF, and morphometry of RLN following crush injury were analyzed. Most previous studies were limited to research on the recovery of vocal cord movement, airway patency, or reinnervation. Using our original method of acoustic wave analysis, our study examined the neglected research area of vocalization recovery, for which we could find no prior relevant studies in RLN crush injury. We used standardized and calibrated methods (e.g., providing equal stimulation intensity, recording the full acoustic range, using equal time intervals following recovery, using equal distance between audio recorders and the mouth) so that the data from the individual animals is reliable and comparable.

After crush injury, there was a full functional recovery of vocalization at 16 weeks. The acoustic area percentage showed a gradual increase as a function of recovery time, and reached a normal level by week 16. The amplitude percentage did not recover by week 3, 6, or 10, though, by week 16, the experimental rats could vocalize normally.

Immediately following RLN injury, all rats scored “0”, which reflected the absence of vocal fold movement. At post-injury week 3, the open and closed angles between arytenoid cartilages remained different from that of the control group (P<0.01), though, we observed a gradual recovery in vocal fold movement after RLN crush injury over a period of 16 weeks. By post-injury week 16, all animals showed complete recovery of motion, which was reflected in the size of the open and closed angles between arytenoid cartilages. Although spontaneous reinnervation restored the voice and full vocal fold movement by post-injury week 16, the vocalization may not only depend on the restoration of the angles between arytenoid cartilages, but may also depend on the recovery of vocal fold tonus [53]. Because the distance over which RLN motoneurons transport post-injury axonal regeneration proteins and the rate of nerve regeneration are different between rats and humans, humans require more time to regenerate peripheral nerves [54]. Thus, the two factors of travel distance for regenerative proteins and nerve regeneration rate may decide the time that is required for full functional recovery [41].

Following RLN injury, degeneration of axons reduces the nerve cell’s capacity for action potentials. In addition, it is well known that the larger the axonal diameter, the stronger its regeneration capability [55]. In our study, at post-injury week 16, the experimental animals' nerves appeared to be structurally normal, and the numbers of axons showed no significant differences in this group than in the control group. Perhaps because the large-diameter axons recovered by post-injury week 6, and the small-diameter axons did not recover, the average-diameter axon recovered its pre-injury diameter by post-injury week 10. Because axons’ numbers of different diameters were various and there was a difference in the recovery rates of different-diameter axons, the capability for self-repair was influenced by various factors. The results suggest that in order to achieve a deeper understanding of recovery rate, future research should evaluate the nerve conduction velocity and toluidine blue staining of nerves.

BDNF contributes to neuronal survival, axon growth, and neuroplasticity [56], and GDNF appears to prevent the degeneration of injured nerves by reducing the motoneuron damage and inhibiting activity of nitric oxide synthase [57]. In this research, we chose to study changes in the gene expression of these two neurotrophic factors. In our study, the TA and PCA muscles showed a little difference in expression of BDNF. Their expressions were increased in the right TA and PCA muscles from week 3, while the increment was obviously in the right TA but not obviously in the right PCA between week 6 and week 10. Although the general variation trend of BDNF and GDNF is similar in the right TA and PCA muscles, they are still different from each other. BDNF expression at post-injury week 3 reached a minimum level and then increased gradually. This change in gene expression may be attributable to a stress response to the surgical operation, which can trigger catabolism in muscles [58]. Further research may allow us to verify this phenomenon shortly. The similarity between the change characteristics of neurotrophic fac-
tors and the pathological changes of injured nerve indirectly showed that neurotrophic factors expression level was closely related to the nerve regeneration. The differences between TA and PCA also may be associated with other post-surgical responses. For example, TA muscles are primarily comprised of fast-twitch fibers, whereas nearly half of PCA muscle fibers are the slow type, thus, these differences in muscle fiber composition may help explain differences in reinnervation [21]. Reinnervation of fast-twitch fibers require fewer muscle fibers than slow-twitch fibers [53]. In the present study, we deduce that the differences in neurotrophin expression may be a function of a preferential reinnervation of adductor muscles. The phenomenon could explain the adducted position of the vocal fold after RLN injury. This explanation seems more plausible than the hypothesis that the adductor and abductor forces cancel each other out, resulting in no net motion. Because of the enhancement of glottis closure, any preferential reinnervation of TA muscle may help patients with unilateral paralysis but not help patients with bilateral paralysis. Further study is imperative to focus on the changes in other neurotrophins and improving the function of PCA muscle in patients with bilateral paralysis. At the same time, the horizontal division of PCA and the superior medial division of TA are mostly composed of slow-type fibers, while the lateral division of TA and the oblique and vertical divisions of PCA have a greater percentage of fast-type fibers, so a study to differentiate the muscle styles would be useful to increase our understanding of recovery of TA and PCA muscle function [59]. Because of the specific functions of BDNF versus GDNF, their gene expression is different [22], so it may be possible to determine how to differentially apply neurotrophic factors to better promote nerve regeneration.

The present study has some limitations that bear consideration. First, we used immunohistochemistry to document the expression of GDNF and BDNF proteins within muscles, and this is valuable for localizing specific proteins in tissues. However, studies conducted using more quantitative techniques (e.g., western blot) should be done to confirm and expand our results. Second, this study was conducted over 16 weeks with a small number of rats, and, thus, larger-scale, as well as shorter- and longer-term studies are needed to confirm our findings. Third, in this pilot study, we performed unilateral crush injury as an animal model of nerve regeneration and functional muscle recovery, so it is important that future studies be conducted that implement bilateral nerve injury. Finally, crush injury also is not representative of more severe types of clinical injury. Further studies are therefore warranted that involve a more severe type of injury of RLN (e.g., transection).

Conclusions

Spontaneous reinnervation occurs following recurrent laryngeal nerve (RLN) injury. To promote the repair of neurological function, neurotrophic factors can be utilized after RLN injury. Strategies to promote sufficient reinnervation of PCA muscle may improve the effectiveness of spontaneous recovery after RLN injury.

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Disclosure of conflict of interest

None.

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