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Immunohistologic Analysis of Spontaneous Recurrent Laryngeal Nerve Reinnervation in a Rat Model

Andrew J. Rosko, MD[†]; Robbi A. Kupfer, MD[†]; Sang S. Oh, DVM, MS; Catherine T. Haring, MD; Eva L. Feldman, MD, PhD; Norman D. Hogikyan, MD

Objective: After recurrent laryngeal nerve injury (RLN), spontaneous reinnervation of the larynx occurs with input from multiple sources. The purpose of this study was to determine the timing and efficiency of reinnervation across a resected RLN segment in a rat model of RLN injury.

Study Design: Animal study.

Methods: Twelve male 60-day-old Sprague Dawley rats underwent resection of a 5-mm segment of the right RLN. Rats were sacrificed at 1, 2, 4, and 12 weeks after nerve injury to harvest the larynx and trachea for immunohistologic analysis. The distal RLN segment was stained with neurofilament, and axons were counted and compared to the nonoperated side. Thyroarytenoid (TA) muscles were stained with alpha-bungarotoxin, synaptophysin, and neurofilament to identify intact neuromuscular junctions (NMJ). The number of intact NMJs from the denervated side was compared to the nonoperated side.

Results: Nerve fibers regenerated across the resected RLN gap into the distal recurrent laryngeal nerve to innervate the TA muscle. The number of nerve fibers in the distal nerve segment increased over time and reached the normal number by 12 weeks postdenervation. Axons formed intact neuromuscular junctions in the TA, with 48.8% ± 16.7% of the normal number of intact NMJs at 4 weeks and 88.3% ± 30.1% of the normal number by 12 weeks.

Conclusion: Following resection of an RLN segment in a rat model, nerve fibers spontaneously regenerate through the distal segment of the transected nerve and form intact NMJs in order to reinnervate the TA muscle.

Key Words: Laryngeal reinnervation, recurrent laryngeal nerve, vocal fold paralysis.

Level of Evidence: NA.

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INTRODUCTION

Laryngeal paralysis secondary to recurrent laryngeal nerve injury remains a significant cause of morbidity by impairing voice, airway, and swallowing function. Historically, although laryngeal paralysis was primarily related to extralaryngeal malignancies and thyroid surgery, the etiology of laryngeal paralysis has been changing in recent decades, with an increase in recurrent laryngeal nerve (RLN) injury during anterior cervical spine surgery, carotid endarterectomy, and thoracic surgery.[1–5] Unfortunately, despite being an area of active research, there is no way to reliably restore physiological movement to the paralyzed vocal fold. The available treatment options for vocal fold paralysis consist of static geometric solutions that provide more favorable glottic closure and reinnervation procedures that provide tone to vocal fold musculature.[6]

Unlike most peripheral nerve injuries, clinical or experimental injury to the RLN does not typically result in progressive atrophy or fibrosis of the laryngeal musculature. This phenomenon can be attributed to a propensity of the larynx to undergo spontaneous reinnervation after RLN injury, which has been demonstrated by multiple authors.[7–12] Unfortunately, aberrant reinnervation can lead to synkinesis rather than physiologic motion.[7] Understanding spontaneous reinnervation and the sources of reinnervation has been the subject of multiple studies.[13–18]

Spontaneous laryngeal reinnervation was studied in previously published work using the current rat model of RLN injury. Multiple experimental conditions were employed to explore and delineate various possible reinnervation sources at 3 months postresection of an RLN segment. It was evident through retrograde brainstem neuronal labeling, electromyography, and laryngoscopy that the superior laryngeal nerve (SLN) is a major source of spontaneous reinnervation, and that the RLN contributes as well—although the RLN contribution was less and appeared variable.[18] There was also evidence for central nervous system plasticity. Remarkably, with respect to the RLN specifically in that study, there was

From the Department of Otolaryngology–Head and Neck Surgery, University of Michigan Health Center (A.J.R., R.A.K., C.T.H., N.D.H.); and the Department of Neurology, University of Michigan Medical School (S.S.O., E.L.F.), Ann Arbor, Michigan, U.S.A.

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[†]These authors contributed equally to this work.

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Send correspondence to Andrew J. Rosko, MD, University of Michigan Health System, Department of Otolaryngology–Head and Neck Surgery, 1500 E Medical Center Dr., 1904 TC, SPC 5312, Ann Arbor, MI 48109-5312. E-mail: arosko@med.umich.edu

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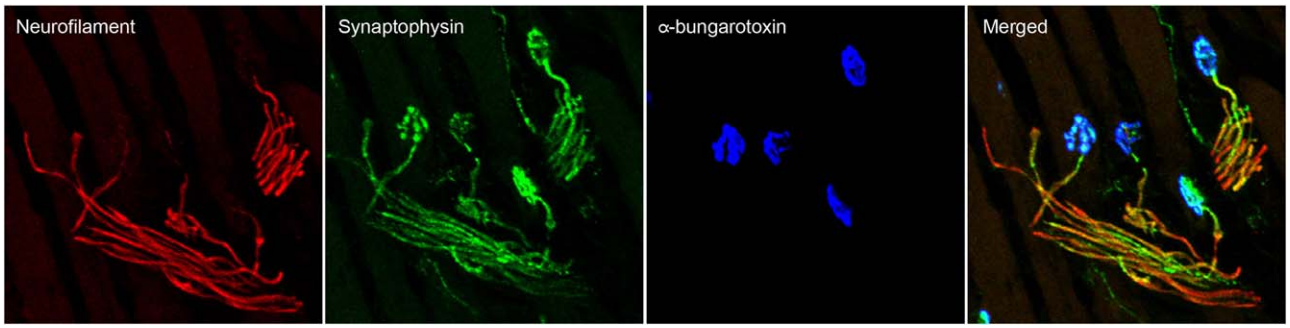

Fig. 1. Immunohistochemical analysis of intact neuromuscular junctions. Neurofilament (red) demonstrates nerve fibers, synaptophysin (green) demonstrates presynaptic terminals, and α-bungarotoxin (blue) demonstrates motor end plates. The three images are merged to assess contact.

histologic evidence of axons in a distal nerve segment across a gap at 3 months postinjury in 87% of animals. It was unclear, however, whether the histologic staining of neurofilament in the distal RLN segment truly represented regenerated axons or was residual from native nerve. It was also unclear whether these axons contributed to functional neuromuscular junctions (NMJ).

We hypothesize that the RLN contributes to spontaneous laryngeal reinnervation by regenerating across a resected gap, and that regeneration of nerve fibers will occur in a time-dependent fashion that correlates with establishment of intact NMJs. The purpose of this study was to evaluate this hypothesis.

MATERIALS AND METHODS

Animals and Procedures

The study was performed with the approval of the University of Michigan Committee on Use and Care of Animals. Twelve male Sprague-Dawley rats, age 60 days, underwent RLN resection using procedures previously described in a rat model of chronic RLN injury.[19] Briefly, intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) with inhaled 1.8% isofluorane were used to induce anesthesia with spontaneous respiration for microlaryngoscopy to document vocal fold mobility. The larynx, trachea, and right RLN were then exposed under an operating microscope through a midline cervical incision, and 5 mm of the right RLN at the level of the cervical trachea was resected. The wound was closed, and microlaryngoscopy was

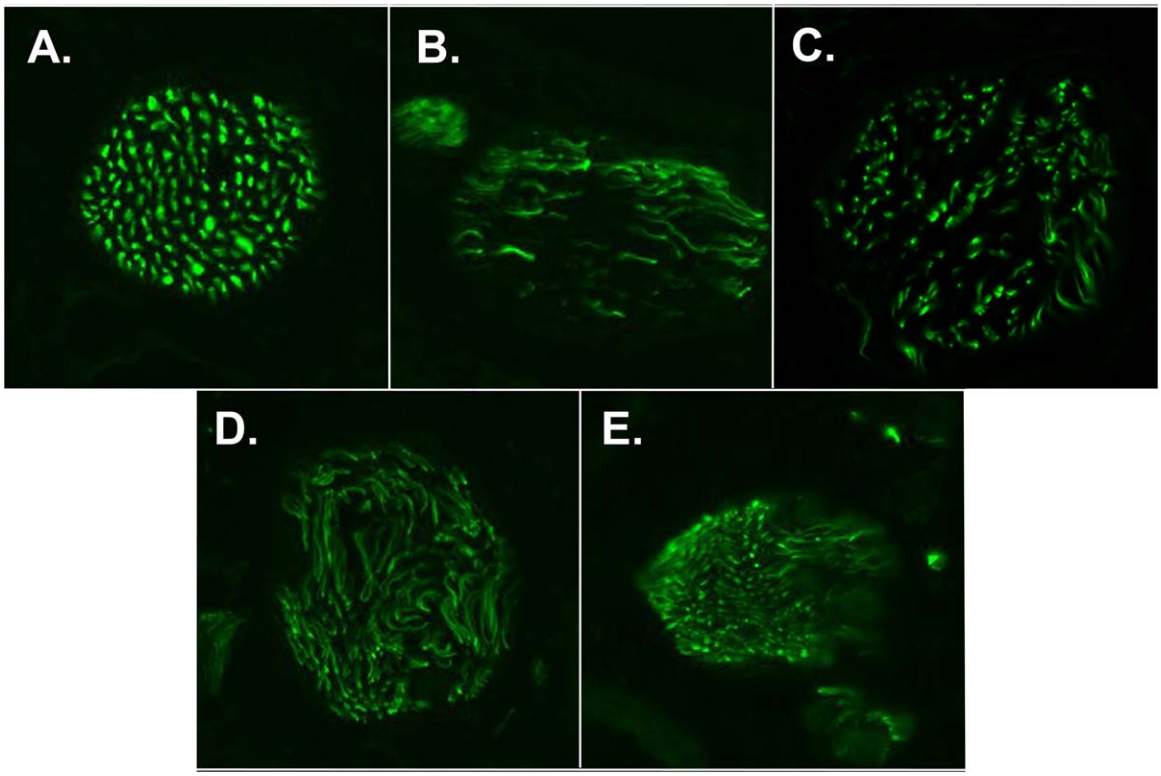

Fig. 2. Immunohistologic analysis with neurofilament stain of the recurrent laryngeal nerve (RLN) across a resected segment. (A) Intact RLN; (B) Neurofilament within RLN location 1 week following resection, (C) 2 weeks following resection, (D) 4 weeks following resection, and (E) 12 weeks following resection.

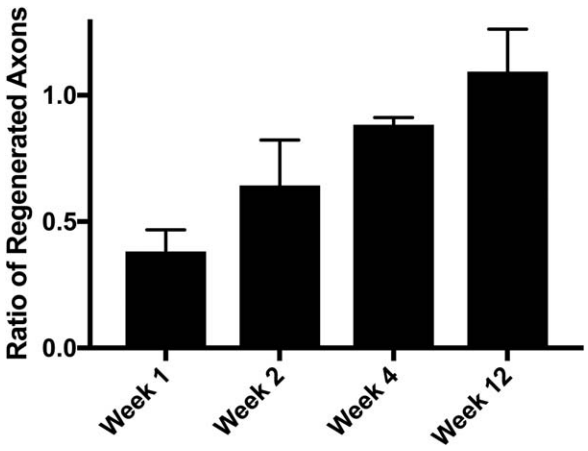

Fig. 3. Axonal counts from the resected section of the recurrent laryngeal nerve over time following nerve injury. Axon counts are shown as a ratio of the injured to uninjured side. Error bars represent standard deviation.

again performed to confirm right vocal fold paralysis. The rats were then divided into four groups of three rats each and harvested at different time points following RLN denervation. Rats were sacrificed at 1 week, 2 weeks, 4 weeks, and 12 weeks.

Harvest and Tissue Processing

Rats were euthanized, followed by intracardiac perfusion of 4% paraformaldehyde. The larynx and trachea with associated RLNs were harvested and sectioned at 20 μm. Immunohistochemistry was then performed. Sections through the trachea and RLNs were processed with anti-neurofilament antibodies (Millipore, Billerica, Massachusetts) to visualize axons within the RLN distal to the resected segment of the nerve. Sections through the resected gap were also examined to confirm that the axons were regenerating across the gap from the proximal nerve segment. A Nikon Microphot FXA microscope (Nikon, Inc., Minato, Tokyo, Japan) was used with a Spot RT slider CCD 3 MP camera and a 40× objective (Diagnostic Instruments, Inc., Sterling Heights, Michigan), providing a total of 400× magnification. Images were acquired using Spot Basic version 4.6.3.10 (Diagnostic Instruments). Images imported into MetaMorph version 7.7.1 (Molecular Devices Corp., Sunnyvale, California), and axons were counted. Four sections were analyzed for each animal. The results from the injured side were compared to the nonoperative side, which served as an internal control. Results were reported as a ratio of the transected side over the normal nonoperative side.

Sections through the thyroarytenoid (TA) muscle were processed with anti-neurofilament antibodies, anti-synaptophysin antibodies (Sigma, St. Louis, Missouri), and α-bungarotoxin (Invitrogen, Carlsbad, California) to visualize the axons, presynaptic terminals, and acetylcholine receptors, respectively, for identification of intact NMJ. A neuromuscular junction was counted if there was triple staining with neurofilament, synaptophysin, and α-bungarotoxin (Fig. 1). Images were captured at 200× power using an Olympus FluoView FV500 scanning laser microscope (Olympus Corp., Shinyuku, Tokyo, Japan). The results from the injured side were compared to the nonoperative side, which served as an internal control. Results were reported as a ratio of the transected side over the normal nonoperative side. The researchers analyzing the sections were unblinded.

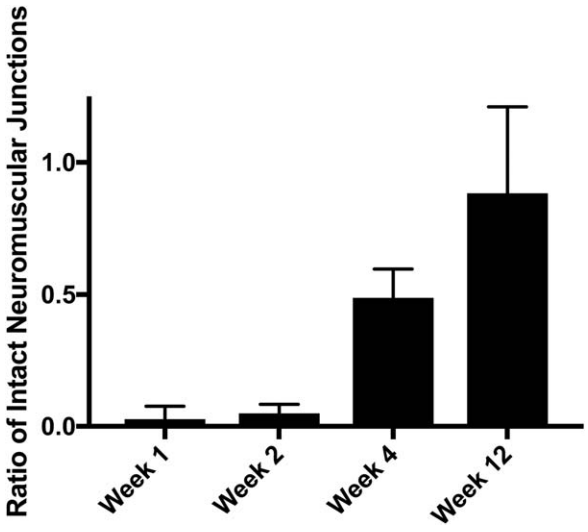

Fig. 4. The number of intact neuromuscular junctions present in the thyroarytenoid muscle were compared over time following recurrent laryngeal nerve injury. The number of intact neuromuscular junctions are shown as a ratio of the injured to the uninjured side. Error bars represent standard deviation.

RESULTS

Animal Procedures

All 12 animals underwent the designated procedures, were confirmed to have right vocal fold paralysis following denervation, survived to the chosen endpoint, and were successfully harvested.

Axon Regeneration Across Resected Segment

There was immunohistochemical evidence of axon regeneration into the distal RLN to innervate the TA muscle (Fig. 2). Neurofilament was present as early as 1 week, and the number of nerve fibers in the distal nerve segment increased over time. At 1 week, 38.2% ± 8.5% of the intact nerve fibers were histologically present across the cut nerve gap. This increased to 64.3% ± 17.9% by 2 weeks and to 88.4% ± 2.8% at 4 weeks, and normalized to 109.4% ± 16.7% by 12 weeks postdenervation (Fig. 3).

Neuromuscular Junction Analysis From the Thyroarytenoid Muscle

Immunohistochemical analysis of NMJs within the TA muscles demonstrated time-dependent increase in the number of intact NMJs following RLN resection. Early in the time course, very few intact neuromuscular junctions were noted on the injured side compared to the normal nonoperated side (2.8% ± 4.9% at week 1 and 4.9% ± 3.5% at week 2). By week 4, the number of intact NMJs increased dramatically to 48.8% ± 10.8% and nearly normalized by 12 weeks (88.3% ± 32.8%) (Fig. 4). Morphologically, the shape of the neurofilament-stained fibers had a more normal appearance as time progressed, and synaptophysin-stained presynaptic terminal increased with time (Fig. 5). Interestingly, the number of α-bungarotoxin-stained motor endplates did not differ between the different time points, but these

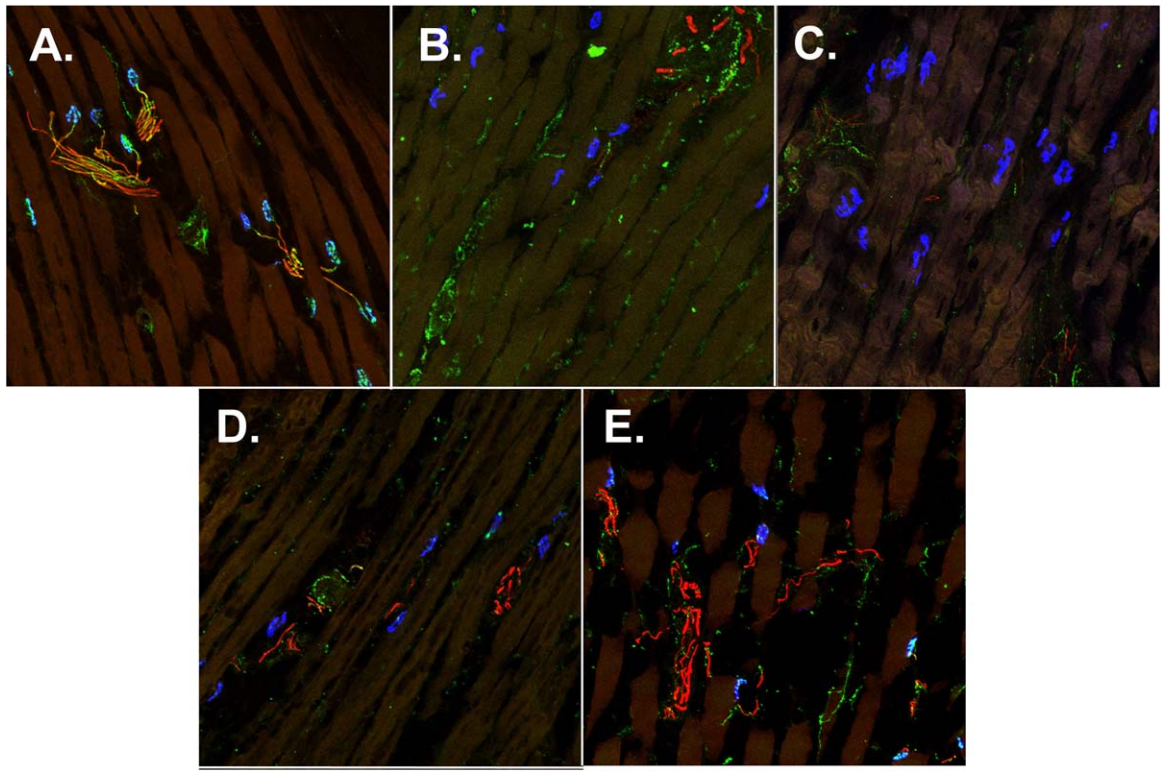

Fig. 5. Immunohistologic analysis of NMJ within the TA muscle. Images are merged to show nerve fibers (red neurofilament stain), presynaptic terminals (green synaptophysin stain), and motor end plates (blue α-bungarotoxin stain). (A) TA muscle with intact NMJ. (B) TA muscle 1 week following nerve injury and (C) 2 weeks following nerve injury, showing normal number of motor end plates but no intact NMJ. (D) TA muscle 4 weeks following nerve injury. (E) TA muscle 12 weeks following nerve injury, showing intact NMJ with altered nerve fiber morphology.
NMJ = neuromuscular junctions; TA = thyroarytenoid.

endplates were not contacted by neurofilament-stained axonal fibers or synaptophysin-stained presynaptic terminals in the earlier time points (Fig. 5B and 5C).

DISCUSSION

As shown in previous studies, there is spontaneous reinnervation of the larynx after RLN injury.[7–12] The current study supports the conclusion that the RLN itself contributes to spontaneous reinnervation. All 12 animals demonstrated regeneration of axons into the distal RLN segment across the resected gap. Interestingly, when the axons regenerated, they were able to identify the distal nerve stump across the unreconstructed nerve gap to reach the larynx. Axon counts in the distal nerve just beyond the injury demonstrated that efficient neural regeneration as axonal density reached near normal numbers by week 4 and had normalized by week 12. The previously published study with this model had demonstrated that 87% of animals had neurofilament staining in the distal nerve segment; however, it was unclear whether the staining was of residual native tissue or was in fact staining regenerated axons. Observing the change in axon count over time in the current study answers this question and is definitive evidence of RLN degeneration and regeneration in the distal nerve segment.

This finding is supported by previous findings. Chen et al. evaluated 29 patients with unilateral vocal fold paralysis after thyroid surgery and explored the recurrent laryngeal nerve. In their study, five patients had intact recurrent laryngeal nerves, whereas 24 had transected nerves. In patients with nerve transection, proliferative connective tissue was seen connecting the proximal and distal ends of the transected nerve. It was postulated that this may indicate spontaneous regeneration of the recurrent laryngeal nerve despite the lack of functional vocal fold movement.[20] Spontaneous axonal regeneration is critical in maintaining tone to the laryngeal musculature after denervation. This has been termed "subclinical reinnervation."[21] The origin of the axons innervating the larynx is often aberrant; however, the current study, along with the findings from Chen et al. and our previous work, suggest that at least some axons originate within the injured recurrent laryngeal nerve.[18,20,21]

The current study also demonstrates the time-dependent formation of intact NMJs in the TA muscle. By 12 weeks, the number of intact NMJs was almost equal to that of the uninjured side. Although the NMJs were very disorganized at early time points, they had a more normal morphological appearance by 12 weeks. These data alone add useful information to the collective knowledge base regarding the phenomenon of spontaneous laryngeal reinnervation, particularly with respect to the possible directed reinnervation paradigms discussed below. In our study, the number of motor endplates did

not change with acute denervation. This is in concordance with the understanding of acute muscle denervation. In the early stage of denervation, the muscle creates a microenvironment that is conducive for reinnervation.[22] This includes upregulation of muscle regulation factors, increase in nicotinic acetylcholine receptors, and Schwann cell proliferation.[22–27] As in our study, the motor end plates remain present, are often upregulated in the acute postdenervation phase, and are a key contributor to successful reinnervation.

The timing of intact NMJ formation had a logical temporal relationship to axonal regrowth in the distal RLN, which further supports the conclusion that the regenerated RLN contributes to the functional regenerated NMJs. This answers a second important question remaining after the previously published study regarding the functional significance of regenerated RLN axons. We do acknowledge that the current experimental paradigm does not allow for delineation of what percentage of the functional NMJs are innervated by RLN versus SLN or other neural sources, but the temporal relationship described above supports an RLN contribution. Consideration was given to resecting the SLN on the experimental side in the current study in order to eliminate this neural regeneration source; however, it was felt that this would significantly alter the denervation/reinnervation milieu and would make the study nonrepresentative of the common clinical situation of isolated RLN injury.

Although this study confirms and further characterizes a source of laryngeal reinnervation, the fundamental issue remains that spontaneous reinnervation is not physiologic and often leads to synkinesis. As such, there is much interest in both improving regenerative efficiency and in guiding axonal growth to prevent synkinesis. The concept of correct nerves reaching correct muscles is the most important goal in laryngeal reinnervation research.

Efforts to improve neural regeneration in general after a recognized nerve injury include primary coaptation, autologous nerve grafting, nerve conduits using an extracellular matrix, bone marrow-derived cells to improve vascularity, supportive cells, gene therapy with growth factors, and even 3D printed scaffolds.[28–34] Although some of these techniques hold promise in terms of promoting axonal growth, they do not address the problem of synkinesis. Selectively promoting desired reinnervation sources and targets and/or preventing undesired ones using growth factors or neurotoxins is an important current arena of investigation, and elegant work is being done in the laboratories of Halum, Paniello, Pitman, and others. [35–37] A thorough characterization of spontaneous reinnervation sources and mechanisms is a necessary prerequisite to achieving the goals of physiologic reinnervation and improved outcomes for patients with laryngeal paralysis.

CONCLUSION

This study demonstrates that axons are able to spontaneously regenerate across a 5-mm gap in a rat model of RLN injury. These axons reach the larynx and contribute to intact neuromuscular junctions. The numbers of regenerated axons and neuromuscular junctions increase in a time-dependent manner and nearly normalize by 12 weeks postinjury.

BIBLIOGRAPHY

1. Rosenthal LH, Benninger MS, Deeb RH. Vocal fold immobility: a longitudinal analysis of etiology over 20 years. *Laryngoscope* 2007;117:1864–1870.
2. Benninger MS, Gillen JB, Altman JS. Changing etiology of vocal fold immobility. *Laryngoscope* 1998;108:1346–1350.
3. Merati AL, Shemirani N, Smith TL, Toohill RJ. Changing trends in the nature of vocal fold motion impairment. *Am J Otolaryngol* 2006;27:106–108.
4. Feehery JM, Pribitkin EA, Heffelfinger RN, et al. The evolving etiology of bilateral vocal fold immobility. *J Voice* 2003;17:76–81.
5. Prasad VM, Fakhoury R, Helou D, Lawson G, Remacle M. Unilateral vocal fold immobility: a tertiary hospital's experience over 5 years. *Eur Arch Otorhinolaryngol* 2017;274:2855–2859.
6. Erman AB, Kejner AE, Hogikyan ND, Feldman EL. Disorders of cranial nerves IX and X. *Semin Neurol* 2009;29:85–92.
7. Crumley RL. Laryngeal synkinesis revisited. *Ann Otol Rhinol Laryngol* 2000;109:365–371.
8. Sahgal V, Hast MH. Effect of denervation on primate laryngeal muscles: a morphologic and morphometric study. *J Laryngol Otol* 1986;100:553–560.
9. Blitzer A, Jahn AF, Keidar A. Semon's law revisited: an electromyographic analysis of laryngeal synkinesis. *Ann Otol Rhinol Laryngol* 1996;105:764–769.
10. Iroto I, Hirano M, Tomita H. Electromyographic investigation of human vocal cord paralysis. *Ann Otol Rhinol Laryngol* 1968;77:296–304.
11. Hogikyan ND, Johns MM, Kileny PR, Urbanchek M, Carroll WR, Kuzon WM Jr. Motion-specific laryngeal reinnervation using muscle-nerve-muscle neurotization. *Ann Otol Rhinol Laryngol* 2001;110:801–810.
12. Johns MM, Urbanchek M, Chepeha DB, Kuzon WM Jr, Hogikyan ND. Thyroarytenoid muscle maintains normal contractile force in chronic vocal fold immobility. *Laryngoscope* 2001;111:2152–2156.
13. Flint PW, Downs DH, Coltrera MD. Laryngeal synkinesis following reinnervation in the rat. Neuroanatomic and physiologic study using retrograde fluorescent tracers and electromyography. *Ann Otol Rhinol Laryngol* 1991;100:797–806.
14. Crumley RL, McCabe BF. Regeneration of the recurrent laryngeal nerve. *Otolaryngol Head Neck Surg* 1982;90:442–447.
15. Nomoto M, Yoshihara T, Kanda T, Kaneko T. Synapse formation by autonomic nerves in the previously denervated neuromuscular junctions of the feline intrinsic laryngeal muscles. *Brain Res* 1991;539:276–286.
16. Nomoto M, Yoshihara T, Kanda T, Konno A, Kaneko T. Misdirected reinnervation in the feline intrinsic laryngeal muscles after long-term denervation. *Acta Otolaryngol Suppl* 1993;506:71–74.
17. Hydman J, Mattsson P. Collateral reinnervation by the superior laryngeal nerve after recurrent laryngeal nerve injury. *Muscle Nerve* 2008;38:1280–1289.
18. Kupfer RA, Old MO, Oh SS, Feldman EL, Hogikyan ND. Spontaneous laryngeal reinnervation following chronic recurrent laryngeal nerve injury. *Laryngoscope* 2013;123:2216–2227.
19. Old MO, Oh SS, Feldman E, Hogikyan ND. Novel model to assess laryngeal function, innervation, and reinnervation. *Ann Otol Rhinol Laryngol* 2011;120:331–338.
20. Chen D, Chen S, Wang W, Zhang C, Zheng H. Spontaneous regeneration of recurrent laryngeal nerve following long-term vocal fold paralysis in humans: histologic evidence. *Laryngoscope* 2011;121:1035–1039.
21. Crumley RL. Experiments in laryngeal reinnervation. *Laryngoscope* 1982;92(suppl 30):1–27.
22. Ma J, Shen J, Lee CA, et al. Gene expression of nAChR, SNAP-25 and GAP-43 in skeletal muscles following botulinum toxin A injection: a study in rats. *J Orthop Res* 2005;23:302–309.
23. Kobayashi J, Mackinnon SE, Watanabe O, et al. The effect of duration of muscle denervation on functional recovery in the rat model. *Muscle Nerve* 1997;20:858–866.
24. Lapalombella R, Kern H, Adami N, et al. Persistence of regenerative myogenesis in spite of down-regulation of activity-dependent genes in long-term denervated rat muscle. *Neurol Res* 2008;30:197–206.
25. Weis J, Kaussen M, Calvo S, Buonanno A. Denervation induces a rapid nuclear accumulation of MRF4 in mature myofibers. *Dev Dyn* 2000;218:438–451.
26. Zhao C, Veltri K, Li S, Bain JR, Fahnestock M. NGF, BDNF, NT-3, and GDNF mRNA expression in rat skeletal muscle following denervation and sensory protection. *J Neurotrauma* 2004;21:1468–1478.
27. Wu P, Chawla A, Spinner RJ, et al. Key changes in denervated muscles and their impact on regeneration and reinnervation. *Neural Regen Res* 2014;9:1796–1809.
28. Lundborg G. A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J Hand Surg Am* 2000;25:391–414.

29. Millesi H. Techniques for nerve grafting. *Hand Clin* 2000;16:73–91, viii.
30. Wang Y, Li ZW, Luo M, Li YJ, Zhang KQ. Biological conduits combining bone marrow mesenchymal stem cells and extracellular matrix to treat long-segment sciatic nerve defects. *Neural Regen Res* 2015;10:965–971.
31. de Luca AC, Lacour SP, Raffoul W, di Summa PG. Extracellular matrix components in peripheral nerve repair: how to affect neural cellular response and nerve regeneration? *Neural Regen Res* 2014;9:1943–1948.
32. Yamakawa T, Kakinoki R, Ikeguchi R, Nakayama K, Morimoto Y, Nakamura T. Nerve regeneration promoted in a tube with vascularity containing bone marrow-derived cells. *Cell Transplant* 2007;16:811–822.
33. Wang D, Liu XL, Zhu JK, et al. Bridging small-gap peripheral nerve defects using acellular nerve allograft implanted with autologous bone marrow stromal cells in primates. *Brain Res* 2008;1188:44–53.
34. Yurie H, Ikeguchi R, Aoyama T, et al. The efficacy of a scaffold-free Bio 3D conduit developed from human fibroblasts on peripheral nerve regeneration in a rat sciatic nerve model. *PloS One* 2017;12: e0171448.
35. McRae BR, Kincaid JC, Illing EA, Hiatt KK, Hawkins JF, Halum SL. Local neurotoxins for prevention of laryngeal synkinesis after recurrent laryngeal nerve injury. *Ann Otol Rhinol Laryngol* 2009;118:887–893.
36. Paniello RC. Vocal fold paralysis: improved adductor recovery by vincristine blockade of posterior cricoarytenoid. *Laryngoscope* 2015;125:655–660.
37. Hernandez-Morato I, Tewari I, Sharma S, Pitman MJ. Blockade of glial-derived neurotrophic factor in laryngeal muscles promotes appropriate reinnervation. *Laryngoscope* 2016;126:E337–E342.