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# Embryologic Innervation of the Rat Laryngeal Musculature—A Model for Investigation of Recurrent Laryngeal Nerve Reinnervation

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**Objectives/Hypothesis:** Optimal management of vocal fold paralysis would entail recurrent laryngeal nerve (RLN) reinnervation resulting in normal vocal fold motion. Unfortunately, RLN reinnervation currently results in a nonfunctional vocal fold due to synkinetic reinnervation. Therapeutic interventions that guide regenerating axons back to the appropriate muscle would prevent synkinesis and restore vocal fold and glottal function. The initial step toward developing these therapies is the elucidation of the embryologic innervation of the larynx. This study aimed to identify the age of occurrence, timing, and pattern of embryologic innervation of the rat larynx, hypothesizing that differences in these parameters exist between distinct laryngeal muscles.

**Study Design:** Descriptive anatomic study.

**Methods:** The larynx of rats aged embryologic day (E) 15, 16, 17, 19, and 21 were harvested and then sectioned. Two rats were used for each age. Sections were colabeled with neuronal class III $\beta$-tubulin polyclonal antibody to identify the presence of axons and alexa 488 conjugate $\alpha$-bungarotoxin to identify the presence of motor endplates. The age at which axons and motor endplates were first present was noted. The position and pattern of the axons and motor endplates was recorded in relation to each other as well as the musculoskeletal anatomy of the larynx. The time at which axons appeared to innervate the medial thyroarytenoid (MTA) muscle, lateral thyroarytenoid (LTA) muscle, and the posterior cricoarytenoid (PCA) muscle was documented.

**Results:** Findings in the rat suggest the RLN reaches the larynx and begins branching by E15. Axons branch dorsally first and reach the PCA muscle before the other muscles. Branching toward the MTA muscle occurs only after axons have reached the LTA muscle. By E19, RLN axons have been guided to and selected their respective muscles with formation of neuromuscular junctions (NMJs) in the PCA, LTA and MTA muscles, though the formation of NMJs in the MTA muscle was comparatively delayed.

**Conclusions:** This study describes the embryologic innervation of the rat larynx and suggests that there are distinct differences in the age of occurrence, timing, and pattern of innervation of the PCA, LTA, and MTA muscles of the rat. These findings lay the foundation for studies investigating the role of guidance cues in RLN axon guidance and the utility of these cues in the treatment of RLN injury via the stimulation of functional, nonsynkinetic reinnervation.

**Key Words:** Recurrent laryngeal nerve, nerve reinnervation, vocal fold paralysis, rat, embryology, innervation, axon guidance.

**Level of Evidence:** N/A.

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## INTRODUCTION

Recurrent laryngeal nerve (RLN) injury is a significant complication of cervical and thoracic surgery. It results in significant patient morbidity and occasionally mortality. Though there are static surgical procedures for rehabilitation, an optimal treatment does not exist, and the ultimate goal of restoring normal vocal fold function has been elusive. To this end, a significant amount of research in RLN reinnervation has been performed in the rat, which is an ideal model of this disorder. This study aimed to identify the age of occurrence, timing, and pattern of embryologic innervation of the rat larynx, hypothesizing that differences in these parameters exist between distinct laryngeal muscles. Findings of this study will lay the foundation for future research in RLN axon guidance.

### *Clinical Background*

During surgery, the RLN may be traumatized by devascularization, stretch, crush, or transection. Unfortunately, permanent unilateral vocal fold paralysis is not an uncommon complication of surgery, occurring in approximately 2% of thyroidectomies, carotid endarterectomies, and open heart surgeries. The percentages in thoracic and skull base surgery are much higher.[1]

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In unilateral vocal fold paralysis, the attendant morbidity of aspiration and dysphonia makes surgical rehabilitation necessary. The best rehabilitation treatments available consist of operations that position an immobile vocal fold in the midline so that glottic closure occurs with adduction of the functional vocal fold. Presently, RLN reanastomosis results in synkinetic reinnervation, which is not successful in restoring vocal fold motion. Reinnervation results in either an immobile vocal fold or nonpurposeful, ineffective movement of the vocal fold.[2,3] Bilateral paralysis results in airway obstruction that may require the life saving treatment of a tracheotomy or cordotomy, each with its own morbidity. It is clear that current treatments available for vocal fold paralysis are suboptimal. Ideal treatment would entail RLN anastomosis with abundant, nonsynkinetic, selective reinnervation of the abductor and adductor muscles, resulting in purposeful vocal fold motion with restoration of normal glottic function.

### *Recurrent Laryngeal Nerve Reinnervation in Humans*

In humans, RLN reinnervation is naturally robust without therapeutic intervention. Reinnervation of the laryngeal muscles is almost always present despite the severity of the injury, and the RLN has shown a strong propensity to reinnervate, even in the worst circumstances. This has been documented in humans after exploration of the RLN months to years after transection without anastomosis.[4,5] It is also supported by the fact that laryngeal electromyography (EMG) performed a few months after injury will always detect voluntary motor unit action potentials (MUAPs), denoting the presence of reinnervation. In addition, the laryngeal muscles are very accepting of reinnervation as is shown by the success of ansa cervicalis-RLN reinnervation months to years after injury.[6,7] After a significant injury, evaluation of EMG MUAPs reveals synkinetic reinnervation, where synkinesis is defined as significant EMG activity in the adductor muscles on attempted abduction and EMG activity in the abductor muscles on attempted adduction. This pattern of innervation, regardless of the amount of axon regeneration (whether or not stimulated by growth factors), will result in nonfunctional vocal folds.[2,3,8,9]

### *Recurrent Laryngeal Nerve Reinnervation in Experimental Animals*

Significant research focused on improving RLN reinnervation has been performed in animal models. The majority of this research has investigated growth factors shown to be neuroprotective or regenerative in other nerves and studying their effects on the RLN and laryngeal motoneurons (RLMNs). Substances used thus far include insulin-like growth factor 1, glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor, and (1R)-1-benzo[b]thiophen-5-yl-2[2-(diethylamino)ethoxy] ethan-1-ol hydroxychloride (T-588).[10–13] These studies have all shown positive effects resulting in augmented axonal regeneration.

Despite findings in the above studies, it is now clear that in experimental animals, RLMNs survive after RLN axonal injury, and regeneration of the RLN is robust without any intervention at all. Recent animal studies have shown that the barrier to functional reinnervation is not insufficient reinnervation but synkinetic reinnervation.[8,14–17] A rat model of RLN has been established, in which rigid endoscopy and transoral laryngeal EMG can be employed serially to follow a standardized RLN injury.[18] This model has been used to study the histologic and electromyographic evolution of a standardized nerve crush injury modeling neuropraxia and axonotmesis.[19] It was also employed to characterize the natural evolution of RLN transection and anastomosis in the rat model using histologic, electromyographic, and kinesiologic end points.[8] The results of this study suggested that without any therapeutic intervention, reinnervation occurs over 16 weeks and is robust in all cases. In all rats where synkinesis was present, as confirmed by EMG evaluation, vocal fold motion was not observed. In those rats with minimal to no synkinesis, vocal fold motion was seen. At 16 weeks, the nerve distal to the anastomosis appeared histologically normal. Over this same time period, MUAPs potential morphology was witnessed to evolve from fibrillations to preinjury baseline. Similar findings were noted in the feline after transection of the RLN.[15] In this study, the findings were not as robust, but significant reinnervation occurred even without nerve reanastomosis. Investigation into the survival of rat RLMNs demonstrate their survival after RLN transection.[16] Furthermore, they survive after transection, anastomosis, and maturation of reinnervation at 16 weeks. The anastomosis site does not significantly impede axonal regeneration, with most of the axons traversing the anastomosis into the distal nerve.[14] The findings of these studies support the hypothesis that natural vocal fold reinnervation is robust but results in vocal fold immobility due to synkinetic reinnervation and not due to insufficient reinnervation.

### *Nerve Guidance*

Considering the above findings in both human and animal models, we propose that the most important challenge to vocal fold rehabilitation via RLN reinnervation after injury lies not in augmenting axonal reinnervation or RLMN survival but in promoting selective, nonsynkinetic reinnervation via nerve guidance (see Appendix). Recognizing this, it then becomes a fundamental necessity to understand how RLN axons are guided through the periphery and then select their target. Nerve guidance is a complex issue that is only beginning to be revealed.

Most research on nerve guidance has been performed in the central nervous system, and only recently have investigations begun in the peripheral nervous system. More specific investigation of cranial nerve axon guidance is in its infancy, and the rules that govern branchial motor axon guidance appear different from those of the spinal motor axon limb system.[20] In general, axon guidance can be separated into long-range axon

guidance and short-range synaptic selectivity. Axon growth cones are sensitive to extracellular guidance cues that steer the growth cone via attractive or repulsive forces.[21] Changes in direction are mediated by microtubule-actin filament interaction and reorganization.[22,23] In the peripheral nervous system during embryogenesis, motor neuron pools have a communal responsiveness to guidance cues based on shared transcriptional programs. As a result, they respond to the cues in a similar fashion, ultimately projecting to the same muscle with high accuracy.[24] Much of the guidance depends on characteristics of the growth cone and how it responds to the guidance cues. To complicate matters, the growth cones' responses to cues are plastic over time and space.[25] For instance, patterned and timed innervation occurs in oculomotor nerve innervation of the extraocular muscles.[26] As the axons extend to their furthest muscle, the ventral oblique, they bypass the three other target muscles. The axons skirt between the forming lateral and dorsal recti and through the ciliary ganglion near its nasal surface. Only after nearing the ventral oblique does the nerve start to branch toward the other muscles, which it previously bypassed. One could surmise that attractive cues from the ventral oblique attract the growing axon. Once the axon reaches the ventral oblique, changes may occur in the ventral oblique or oculomotor nerve to sensitize the axons to attractant factors in the other muscles, initiating axonal branching in their direction. Another example is that of retinal axon response to netrin-1 changes from attraction to repulsion as it progresses along its pathway. This change is coordinated with a decrease in 3′-5′-cyclic adenosine monophosphate (cAMP) in the growth cone and is reversed by artificially raising the cAMP.[27] How the axons of the RLN are guided to the correct muscles during embryogenesis is unknown.

To our knowledge, only one study has investigated RLN axon guidance.[28] Vega-Cordova et al. investigated the expression of nerve growth factor (NGF) in abductor and adductor muscles after RLN transection in the mature rat, looking for disparities between the muscles. They noted variable expression of NGF in the thyroarytenoid muscle and GDNF in the posterior cricoarytenoid muscle. They hypothesized that these differences may be partially responsible for preferential RLN reinnervation of the posterior cricoarytenoid (PCA) muscle in the feline.

Considering the above, to achieve the goal of functional RLN reinnervation we must prevent synkinetic reinnervation by guiding the regenerating axons to the correct muscles. The first step toward this goal requires an understanding of axon guidance during embryologic innervation of the larynx. The specific aim of this study was to identify the age of occurrence, timing, and pattern of embryologic innervation of the rat larynx and to test the hypothesis that differences in these parameters exist between distinct laryngeal muscles.

## MATERIALS AND METHODS

### *Surgery*

The larynx of rats embryologic day (E) 15, 16, 17, 19, and 21 were harvested and then sectioned. Two rats were used for each age. The choice of these ages was based on findings of the gross musculoskeletal development of the rat larynx.[29] Five pregnant female Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) were used in this study. Animals with the appropriately aged embryos were sedated with isoflurane (Baxter, Deerfield, IL) and then anesthetized with an intramuscular injection of a mixture of 70 mg/kg of ketamine (Baxter) and 7 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA) to produce sufficient anesthesia. Into the site of the skin incision 1% lidocaine was injected. 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.), and the animal use protocol was approved by the Institutional Animal Care and Use Committee of New York Medical College. Humane care was provided for these animals, and all institutional and national guidelines were observed. All surgeries were performed using an operating microscope (Carl Zeiss, Oberkochen, Germany). A vertical incision was made to enter the abdominal cavity. The embryonic sac was identified. The sac was entered with sharp dissection, and the fetuses were atraumatically removed. The abdominal cavity of the mother was closed with 4-0 Vicryl suture (Ethicon, Somerville, NJ). The mother rat was then euthanized by lethal inhalation of isoflurane. The embryos were then transected at the mid thorax. Tissue cranial to the transection was placed in phosphate-buffered saline (PBS) (0.1 M, pH 7.4) at 98°F and then washed successively in room temperature and 4°C PBS. Isolated embryos were fixed by immersion in 4% paraformaldehyde in PBS for 1 hour. They were then transferred into 30% sucrose in PBS and kept at 4°C until they submerged to the bottom of the container.

### *Immunohistochemistry*

For evaluation of neuromuscular development and innervation, the embryonic larynx was sectioned at 20 $\mu$m using a Leica cryostast. (Leica Microsystems, Wetzlar, Germany). Laryngeal sections were then colabeled with neuronal class III $\beta$-tubulin polyclonal antibody (BT) (Covance, Princeton, NJ) to identify the presence of axons with and alexa 488 conjugate $\alpha$-bungarotoxin (ABT) (Invitrogen, Grand Island, NY) to identify the presence motor endplates. Sections were washed with 5% tris-buffered saline (TBS) (0.1 M, pH 7.4). They were then transferred to 5% bovine serum albumin (BSA) in TBS for 30 minutes. Sections were then transferred to BT (dilution 1:1000 in TBS plus 0.8% BSA) for 48 hours at 4°C in a moisture chamber. Sections were then brought to room temperature and bathed for 15 minutes twice in 5% normal sheep serum (NSS) in TBS. Sections were then incubated in anti-rabbit immunoglobulin G (IgG) Cy3 conjugate (1:400 in 5% TBS with 0.8% NSS) for 1 hour at 4°C in a moisture chamber. They were then incubated in ABT (1:500 in 5% TBS) for 2 hours at room temperature in a moisture chamber. They were then washed in 5% TBS for 15 minutes four times. Sections were then covered with a glycerin:PBS 1:1 solution and cover-slipped and sealed with nail polish. Slides were stored at 4°C. Controls were treated as above with omission of the addition of either BT or ABT. Sections were visualized and evaluated using a Zeiss Axioskop epi-fluorescence microscope (Carl Zeiss) or Nikon confocal microscope (Nikon, Tokyo, Japan) with specific absorption and reflection filters. Colocalization of the axon and motor endplates (MEPs) was performed by alternating between these filters without moving the microscope stage. Images were captured using a motorized camera and then superimposed on each other utilizing Photoshop (Adobe Inc., San Jose,

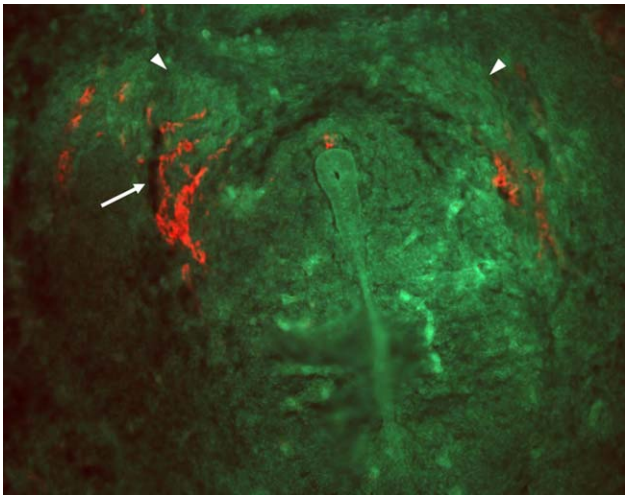

Fig. 1. Embryologic day 15 rat glottis. Axons are labeled red with neuronal class III $\beta$-tubulin polyclonal antibody. Axonal branching is noted and most significant dorsally (arrow). A collection of mesodermal cells are seen lateral and dorsal to the epithelial lamina and are identifiable as the progenitors of the posterior cricoarytenoid muscle (arrow heads). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CA). For confocal microscopy, images were superimposed using the standard confocal imaging methods. The age at which axons and MEPs were first present was noted. The position and pattern of the axons and MEPs was recorded in relation to each other as well as the musculoskeletal anatomy of the larynx. The time at which axons appeared to innervate the medial thyroarytenoid (MTA) muscle, lateral thyroarytenoid (LTA) muscle, and PCA muscle was documented. After it was identified that the majority of innervation took place between E15 and E17, E16 rats were added to the protocol.

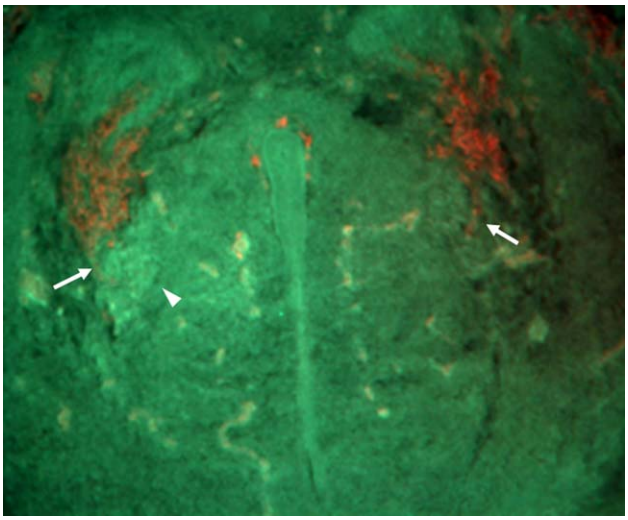

Fig. 2. Embryologic day 16 rat glottis. Neuronal class III $\beta$-tubulin polyclonal antibody stained axons are localized with evidence of early branching. A few ventral branches have now reached the area of the progenitor cells of the lateral thyroarytenoid (arrow). Alexa 488 conjugate $\alpha$-bungarotoxin staining reveals the absence of motor endplates, although there is increased intensity in the area destined to become the lateral thyroarytenoid muscle (arrowhead). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

## RESULTS

### *Embryologic Day 15*

At the glottic level of the E15 rat, the central epithelial lamina is visualized. MEPs are not seen. Axons are labeled with BT and are seen in the lateral aspect of the larynx bilaterally. Axonal branching has begun. This is more significant dorsally and with only minimal branching ventrally. A collection of mesodermal cells are seen lateral and dorsal to the epithelial lamina and are identifiable as the progenitors of the PCA muscle. The

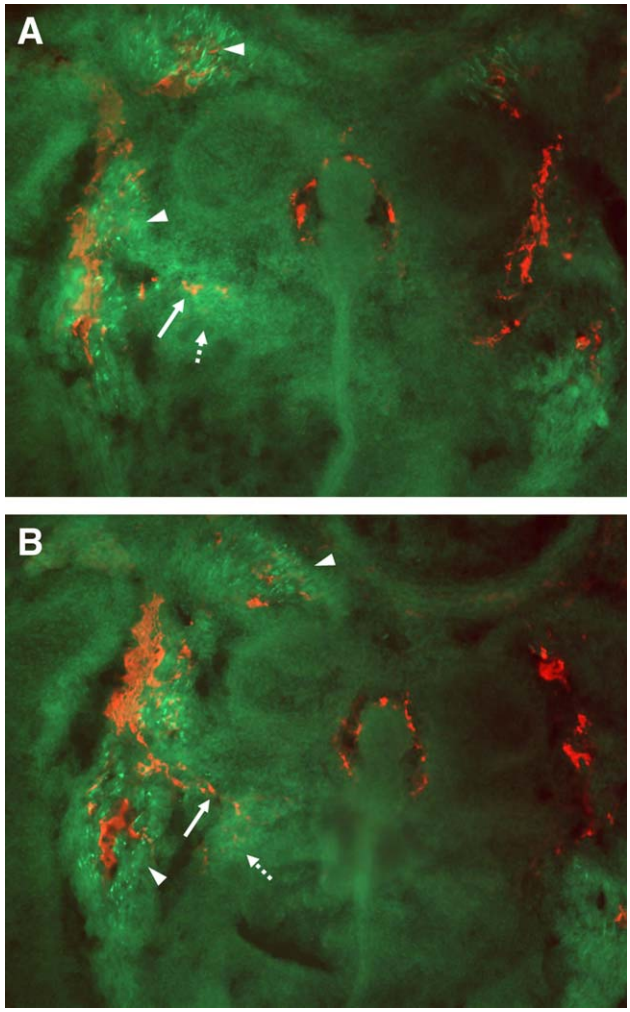


Fig. 3. (A, B) Embryologic day 17 rat glottis, successive sections. Diffuse motor endplates (MEPs) are seen in the posterior cricoarytenoid (PCA) muscle and lateral thyroarytenoid (LTA) muscle (arrowheads). Axons appear to be aggressively infiltrating the PCA and LTA muscles. A smaller amount of axons are seen extending from the ventral branch of the recurrent laryngeal nerve toward the medial thyroarytenoid (MTA) muscle (arrow). Although the area of the MTA muscle is hyperintense with alexa 488 conjugate $\alpha$-bungarotoxin labeling, MEPs are not visualized (dashed arrow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

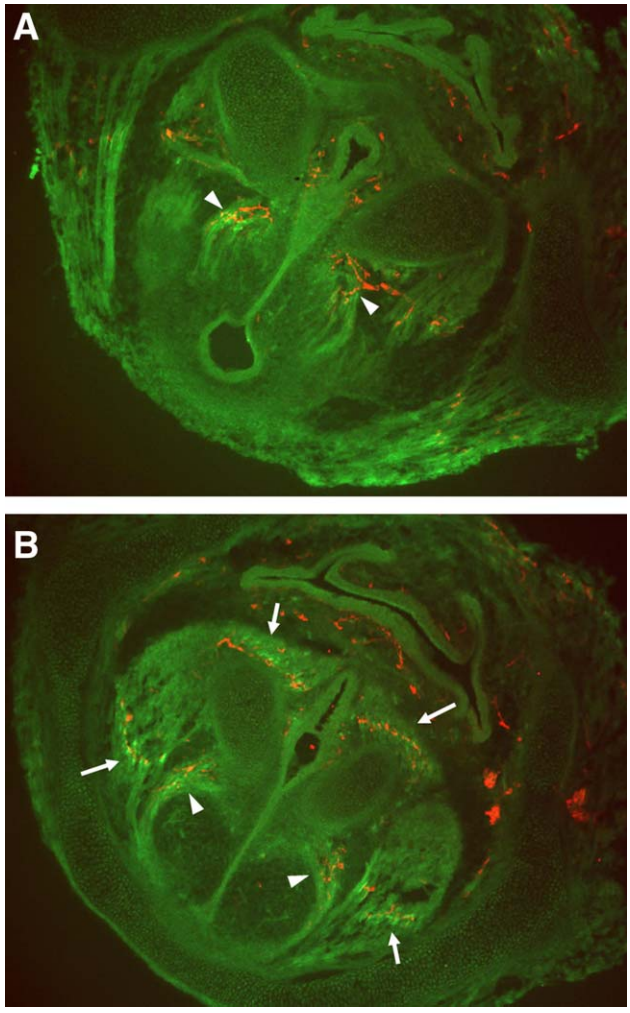


Fig. 4. (A, B) Embryologic day 19 rat glottis, successive sections. The laryngeal muscles, cartilages, and vocal folds are visualized. The motor endplates (MEPs) of the lateral thyroarytenoid muscle and posterior cricoarytenoid muscle have migrated into a central band (B) (arrow). On all sections, the medial thyroarytenoid muscle, although innervated by well-defined branching axons, has yet to evidence medial migration of its MEPs (arrowhead). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dorsal branching axons appear to be moving in their direction (Fig. 1).

### *Embryologic Day 16*

The E16 rat shows minimal progression from the E15 rat. The axons are still localized with evidence of early branching. A few ventral branches have now reached the progenitors cells of the LTA muscle. ABT staining reveals the absence of MEPs, though there is increased intensity in the area destined to become the LTA muscle (Fig. 2).

### *Embryologic Day 17*

There was a tremendous amount of progress between E16 and E17. At E17, copious MEPs are seen in the PCA and LTA muscles. They are diffusely dispersed throughout the muscles, consistent with immaturity. The axons appear to be aggressively infiltrating the PCA and LTA muscles. A smaller amount of axons are seen branching from the ventral branch of the RLN toward the MTA muscle. Although there is a diffuse fluorescent intensity to the MTA muscle with ABT staining, discernable MEPs are not visualized (Fig. 3)

### *Embryologic Day 19*

At E19, the laryngeal muscles, cartilages, and vocal folds have become distinct and easily identifiable. The glottis is partially cannulated dorsally. The ventral

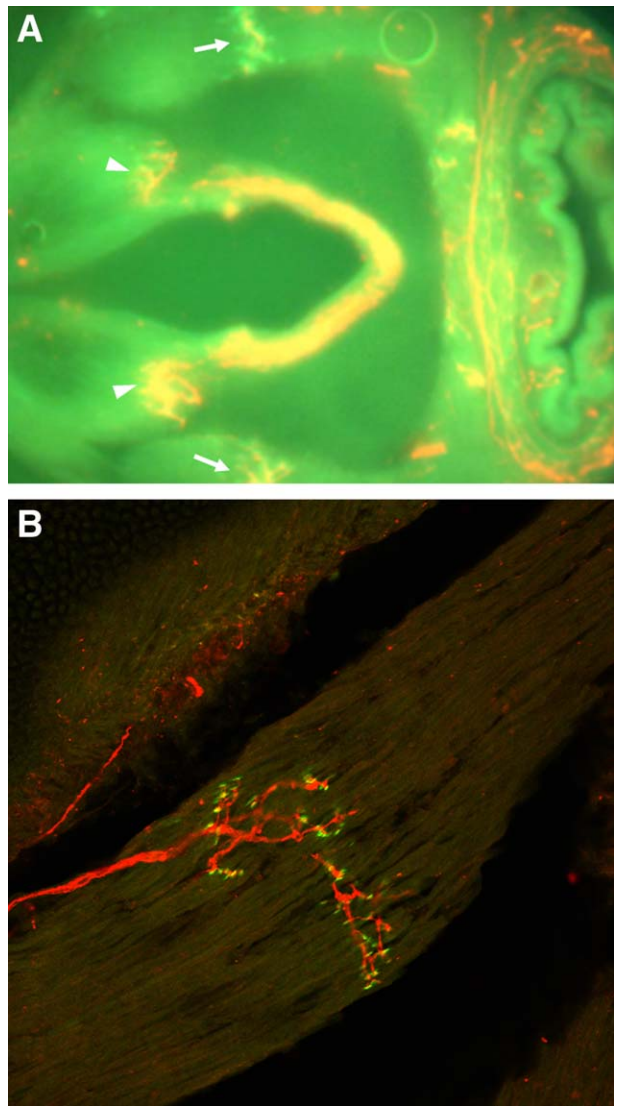


Fig. 5. (A) The gross larynx is fully developed by embryologic day 21. The motor endplates (MEPs) of the lateral thyroarytenoid muscle (arrow), medial thyroarytenoid muscle (arrowhead), and posterior cricoarytenoid (PCA) muscle have all migrated medially. (B) A 60× confocal image reveals formed neuromuscular junctions with a single axon innervating multiple MEPs arranged in a central band within the PCA muscle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

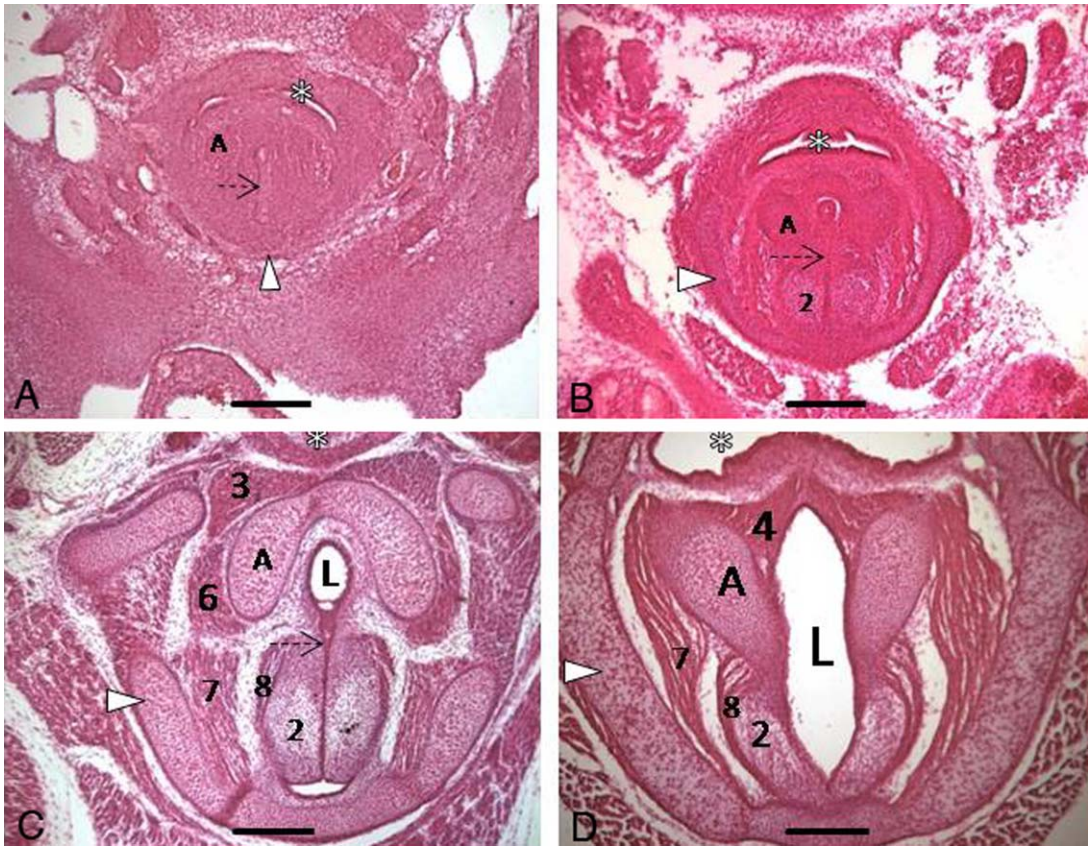


Fig. 6. Larynx at embryologic day (E) 15 (panel A), E17 (panel B), E19 (panel C), and E21 (panel D). The arrow head indicates thyroid cartilage, and the dashed arrow indicates the epithelial lamina. A scale bar in each photograph represents 200 $\mu$m. Modified from Alli et al.[29] A = arytenoid cartilage/swellings; L = lumen of the larynx; 2 = true vocal cord; 3 = posterior cricoarytenoid muscle; 4 = superior cricoarytenoid muscle; 6 = lateral cricoarytenoid muscle; 7 = lateral thyroarytenoid; 8 = medial thyroarytenoid. *Pharynx/esophagus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

paired midline swellings of the primordial true vocal folds are clear. They are boarded laterally by the bellies of the LTA and MTA muscles. Posteriorly located are the developing arytenoids cartilages (ACs) and PCA muscle. The thyroid lamina is seen enveloping the endolarynx. The MEPs of the LTA muscle have migrated into a central band, as have the MEPs of the PCA muscle as seen on a lower section. On all sections, the MTA muscle, though innervated, has yet to evidence medial migration of its MEPs (Fig. 4).

### *Embryologic Day 21*

As expected, the gross larynx is fully developed by E21. The lumen of the glottis is unobstructed. The paired elongated ACs flank the dorsal boarder of the lumen. The MTA, LTA, and PCA muscles are seen. The MEPs of the LTA, MTA, and PCA muscles have all migrated medially. High power 60× confocal images reveal neuromuscular junctions (NMJs), with a single axon innervating multiple MEPs arranged in a central band within the muscle (Fig. 5).

## DISCUSSION

The results from the current study are an important step in the investigation of RLN axon guidance during embryogenesis and reinnervation. Although research of guidance cues in the laryngeal muscles of mature rats after nerve transaction has only just begun, the embryologic innervation of the rat larynx has never been investigated.[28] In fact, the gross musculoskeletal embryogenesis of the rat larynx has only recently been described[29] (Fig. 6). The specific aim of this investigation was to identify the age of occurrence, timing, and pattern of embryologic innervation of the rat larynx, hypothesizing that differences exist between distinct laryngeal muscles. The results of this study suggest that the RLN reaches the larynx by E15. At this time the endolarynx still appears undifferentiated. Axons have reached the PCA muscle progenitor cells with minimal ventral branching. By E16, axons are seen branching ventrally, reaching the LTA muscle. Although the PCA muscle was reached first by axons, ABT labeling appears at this stage in the LTA muscle but not in the PCA muscle. The labeling is diffuse, and MEPs are not seen, but the localized hyperintensity of the ABT is distinct. It appears that axons grow preferentially toward the PCA muscle, at least temporally. This is similar to what occurs during reinnervation, as the PCA is consistently reinnervated first in the rat.[8] The reason for this temporal pattern of innervation and reinnervation is unclear. The presence of guidance cues and differences between their activity in

the PCA and LTA muscles, as well as the growth cone reactivity at this time period, need to be investigated. As the RLN is present and dorsal branching has already occurred at E15, evaluation of earlier stages should be addressed as well.

At E17, the muscles become infiltrated with axons, and the MEPs become more distinct. It is likely that myotubules are forming initial contacts with the axons at this stage. Also at this time, only after the LTA muscle has been innervated do branches to the MTA appear. As was seen previously with the LTA muscle, MTA muscle MEPs are not visible at this stage, but there is a hyperintensity to the MTA muscle progenitor cells with ABT staining. This pattern of axon branching, beginning only after initial innervation of other muscles in the same system, is consistent with what is seen in embryologic innervation of the extraocular muscles.[26] Patterned and temporal innervation suggests a change in guidance and branching cues or the axons' responses to them. In this case, it occurs once the LTA muscle has been innervated. Whether these changes are in the axons, LTA muscle, MTA muscle, or a combination thereof is unknown and is another area for future investigation.

By E19 the NMJs of the PCA and LTA muscles continue to mature as the NMJs migrate into a central band in each muscle.[30] The MTA muscle NMJs are also visualized. They have not formed a distinct central band at this point but are concentrated toward the center of the muscle. This would be expected, as innervation of the MTA muscle occurs later than innervation of the PCA and LTA muscles. Considering that the migration of the MTA muscle NMJs have already begun by E19, it is likely the MTA muscle MEPs appear at E18. At E19, the muscles are maturing, as distinct myotubules or myofibrils are visualized for the first time.

Innervation of PCA, LTA, and MTA muscles reveals centralization of all NMJs by E21. Confocal microscopy of the LTA muscle shows MEPs banding with a single axon branching to innervate multiple NMJs.

## CONCLUSION

Findings in humans and in experimental animal studies suggest that RLMNs survive distal axon injury, RLN axons robustly reinnervate the larynx without intervention, and vocal fold immobility is due to synkinetic reinnervation and not due to insufficient reinnervation. For reinnervation to result in normal vocal fold function, axons must be guided back to the muscle they originally innervated. To facilitate this, we are compelled to investigate and understand the guidance of RLN axons in the periphery.

The initial step toward this goal is to elucidate the embryologic innervation of the larynx. This study describes the embryologic innervation of the rat larynx and suggests that there are distinct differences in the age of occurrence, timing, and pattern of innervation of PCA, LTA, and MTA muscles of the rat. These findings lay the foundation for studies investigating the role of guidance cues in RLN axon guidance and the utility of these cues in the treatment of RLN injury via the stimulation of functional, nonsynkinetic reinnervation.

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## APPENDIX I: BACKGROUND – AXON GUIDANCE LITERATURE REVIEW

To understand why reinnervation is synkinetic and how we may correct it, we must learn how axons are guided through the periphery and then select their target.

Investigations of reinnervation after nerve injury in the mature rat have long suggested recapitulation to embryogenesis during nerve regeneration after nerve injury in the central and peripheral nervous system. This has been evidenced in the upregulation of developmental proteins and the down regulation of proteins associated with mature neurons in Schwann cells and oligodendrocytes as well as motor and sensory axons.[A1–A9] These findings are not always consistent as disparities exist between the embryonic and reparative states.[A1,A9] Nevertheless, it is likely there is much to learn from how innervation occurs correctly in embryogenesis. In addition, differences between embryologic innervation and reinnervation in the mature mammal may suggest new avenues to explore in the search for therapies that may result in the successful treatment of RLN injury.

The developing embryo has been used extensively to study nerve guidance during innervation. In general, axon guidance can be separated into axon guidance and synaptic selectivity. Axon growth cones are sensitive to extracellular guidance cues that steer the growth cone via attractive or repulsive forces acting over a long range (a few millimeters) or a short range.[A10] Changes in direction are mediated by microtubule-actin filament interaction and reorganization.[A11,A12] In the peripheral nervous system during embryogenesis, motor neuron pools have a communal responsiveness based on shared transcriptional programs so that they respond to guidance cues in a similar fashion, ultimately projecting to the same muscle with high accuracy.[A13] Schwann cells follow growth cones which provide migratory guidance in embryogenesis.[A14] The best understood guidance proteins are netrins, slits, semaphorins and ephrins.[A15] Netrins are evolutionary conserved proteins that can attract and repulse growth cones. Slits are mostly repulsive and are know for their modulation of axon guidance in the midline of the central nervous system. Semaphorins and ephrins can also give bidirectional cues as do the netrins.[A15,A16] There is evidence that neurotrophins NGF,GDNF and BDNF mediate attractive axon guidance.[A17–A19] Much of the guidance depends on characteristics of the growth cone and how it responds to the guidance cues. To make matters more complicated, the growth cones' responses to cues are plastic over time and space.[A15] For instance, retinal axon response to netrin-1 changes from attraction to repulsion as it progresses along its pathway. This change is coordinated with a decrease in cyclic AMP in the growth cone and is reversed by artificially raising the cAMP.[A20]

To date, the majority of research in axon guidance has occurred in the central nervous system with some in the spinal motor axon–limb system of the peripheral nervous system. Only recently has attention turned to axon guidance of the branchial motor cranial nerves. Most of this work has been confined to early axon guidance in the brainstem as well as axon emergence from the brainstem. Precious little is known about guidance of such axons in the periphery and as they near their targets.[A21–A22]

In embryogenesis of cranial nerve motorneurons, the hindbrain is divided into rhombomeres which contain motorneurons that ultimately differentiate into the different brainstem nuclei. The vagal branchiomotor nuclei are in rhombomeres 8 and 9 in the chick and mouse. Hox genes play a role in formation and differentiation of the motor neuron bundle of the facial and trigeminal nuclei.[A21] The combination, timing and expression of different Hox genes dictate the identity of each rhombomere. Hox genes may also play a role in early axon guidance and survival via Hox gene dependent differentiation of neural crest cells into Schwann cells. The Schwann cells may provide guidance or survival cues to the axons.[A23,A24]

The initial steps of cranial nerve extension occur in vivo when the nerves are repelled from the floor plate by netrin 1 and slit proteins as well as cadherin 7.[A25,A26] Exit from the hindbrain follows the pathway of sensory ganglia which may exert a chemoattractive influence on branchiomotor axons.[A21]

Guidance cues for extending cranial neurons through the periphery are largely unknown but thought to be a balance of attractive and repulsive cues as is seen with axons of the spinal motor axon–limb system. Semaphorin SEMA3 has been shown to guide the peripheral projection of branchiomotor axons in mice. If the function of SEMA3 is blocked, the axons defasciculate and aberrant axonal projections are observed in multiple cranial nerves.[A27–A28] Recent work shows that cadherin 7 and cadherin 6b also play significant roles in cranial nerve guidance. Cadherin 7 promotes the growth of an unbranched axon away from the floor plate in the brainstem and suppresses formation of multiple axons and branching. As the nerve matures, cadherin 6b stimulates ordered branching near the branchial muscle plate. In vivo studies show that the loss of cadherin function results in aberrant axonal growth.[A26] Neurotrophic factors such as BDNF, NGF, NT3/NT4,CNTF, CT-1and GDNF have also been implicated in trigeminal, facial and vagal cranial nerve guidance but their actions are less understood.[A16,A19,A29,A30]

Even less is known about how axons choose to synapse to a specific muscle once they have been guided to them. Limb axons from common motoneuron pools project to the same set of muscles and then respond to local environmental cues that ensure they synapse to the appropriate muscle.[A13,A31] In the cranial nerve system, it appears that neural crest cells are important in predetermination of the fate of cranial myogenic mesoderm but they are not sole the determinates of muscle differentiation and innervation.[A32] Axons projecting from manipulated rhombomeres to incorrect muscles are eliminated, suggesting target recognition influences.[A33] The methods of action and identity of many of these target

recognition cues are unknown. Ephrins appears to play a significant role as manipulation of ephrin-Eph signaling yields aberrant branching of the trigeminal motor axon.[A34] In the spinal motor axon limb system GDNF expressed by the muscle has been shown to activate Pea3 by a retrograde mechanism and stimulate axonal branching as the axon nears its target.[A35] Patterned and timed innervation occurs in the oculomotor nerve innervation of the extraocular muscles.[A36] As the axons extend to their furthest muscle, the ventral oblique, they bypass the three other target muscles. The axons skirt between the forming lateral and dorsal recti and through the ciliary ganglion near its nasal surface. Only after nearing the ventral oblique does the nerve start to branch towards the other muscles which it previously bypassed. One could surmise that attractive cues from the ventral oblique attract the growing axon. Once the axon reaches the ventral oblique, changes may occur in the ventral oblique or oculomotor nerve to sensitize the axons to attractant factors in the other muscles, initiating axonal branching in their direction.

An excellent review by Sanes and Yamagata suggest ten mechanisms of axon-cell specificity: recognition, guidance, afferent interaction, inhibition, intermediate target utilization, elimination, death, conversion, dendritic choices and proximity/timing.[A37] Though a complete recapitulation of this summary is beyond the scope of this manuscript, there are some interesting points to highlight. Once again, distal recognition molecules are featured. Two classes of cell-surface receptors implicated in target recognition are cadherins and immunoglobulin superfamilies. In addition ephrins, semaphorins and netrins, the same proteins in brainstem guidance, are shown to dictate synaptic specificity. Wnt, Capricious and Toll are also proteins more recently identified as players in axon guidance and target specificity.

Important to our study of synkinetic reinnervation is the inhibition of inappropriate synapses via repellent cues. Semaphorin 2, Toll and Wnt4 have all been implicated in this process. An elegant study by Inaki et al was performed in Drosophila.[A38] They identified Wnt4 as a candidate protein for axon guidance and noted its presence in muscle 13(M13) but not in the adjacent muscle 12(M12). Each muscle is innervated by distinct motor neurons without any crossover between the muscles. When Wnt4 activity was blocked, the nerve innervating M12 decreased its innervation of M12 and formed synapses on M13. When Wnt4 activity was ectopically expressed in M12, synapse formation on M12 was inhibited. These results suggest that Wnt4, in Drosophila, is integral to target specificity via repulsion of axons targeting an inappropriate muscle.

Sanes and Yamagata suggest programmed cell death as a possible, albeit still unsupported, mechanism of synaptic specificity.[A37] This theory suggests that if a muscle innervated by an inappropriate axon fails to excrete retrograde trophic factors the cell will die and the specificity of the remaining axons will sharpen. Specificity would sharpen by increasing the percentage of synapses correctly innervated and by creating empty motor endplates available for continued appropriate innervation.

Though our knowledge of axon guidance in embryogenesis is minimal, even less is know about such guidance during reinnervation. Recent investigations suggest the importance of Schwann cells and Ephrins. Villegas et. al. utilized the zebrafish lateral line mechanosensory system to evaluate axon guidance in reinnervation.[A39] This system has been useful in studying the interactions between axons and Schwann cells in embryogenesis but untested in reinnervation. Their research showed that the absence of Schwann cells after injury resulted in aberrant pathfinding of regenerating axons without influence on the rate of axon growth. Control axons regenerated via their original path. Schwann cells are know to create Bungner bands for regenerating axons to follow and they are also know to illicit neurotrophic factors after axon injury.[A40] Whether the misguided growth of the axons was due to lack of appropriate guidance cues or structural guidance provided by the Schwann cells is unknown. Perrinello et. al. showed that Schwann cells and fibroblasts interact to coordinate axon guidance during regeneration.[A41] This is mediated by ephrin-b/EphB signaling which results in cellular sorting. As a result, Schwann cells precede axon regrowth and create multicellular cords to guide regenerating axons across the injury in a newly formed nerve bridge. When EphB2 signaling is suppressed, axon regrowth is disorganized and multidirectional. Parrinello et. al. also note that in contrast to the majority of embryologic innervation where the Schwann cells follow the growth cone, as the axons approach their target in the final stage of limb innervation, the growth cones become enveloped by the Schwann cells. Citing others work, they hypothesize that Schwann cells may be integral to late axonal branching and targeting during embryogenesis, and therefore regeneration may be a recapitulation of late embryologic innervation.

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