Electromyographic and Histologic Evolution of the Recurrent Laryngeal Nerve From Transection and Anastomosis to Mature Reinnervation

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Objectives: To describe the natural evolution of recurrent laryngeal nerve (RLN) reinnervation in an animal model.

Study Design: Twenty Sprague Dawley rats underwent unilateral RLN transection and anastomosis. Animals were sacrificed at 4, 8, 12, 16, and 20 weeks. Prior to sacrifice, each rat underwent electromyography (EMG) and visual grading of vocal fold motion. Bilateral RLNs were harvested and evaluated histologically.

Results: EMG revealed synkinetic reinnervation at all time periods except at 4 weeks. EMG evolution plateaued at 16 weeks. Vocal fold motion was slight in three rats at 4 weeks but was otherwise absent except for one rat at 12 weeks. Histologic changes of the axons and their myelin sheaths were consistent at each time period. At 16 weeks, histologic changes plateaued.

Conclusions: Consistent EMG, histologic, and vocal fold motion changes occur at specific time periods during RLN reinnervation after transection and anastomosis in a rat model. Reinnervation is mature at 16 weeks. Findings corroborate theories of preferential and synkinetic reinnervation after RLN transection. Use of a rat model to investigate the effect of interventions on RLN reinnervation requires a minimum of 16 weeks between transection and investigation to allow for maturation of reinnervation.

Key Words: Recurrent laryngeal nerve, rat model, vocal fold paralysis, nerve reinnervation.

Level of Evidence: N/A.
Surgery

All surgeries were performed using an operating microscope (Zeiss, Hennigsdorf, Germany). A vertical midline cervical incision was made. The right RLN was exposed in the tracheoesophageal groove and sharply transected with an iridectomy scissor at the seventh tracheal ring. The nerve ends were anastomosed using a common suture free technique utilizing saline hydrated gel foam (Pharmacia & Upjohn, New York, NY) to prevent further nerve trauma from suture anastomosis. Gel foam was placed beneath the nerve prior to transection. When the nerve was transected, there was minimal separation of the nerve ends secondary to their adherence to the underlying gel foam. The gel foam was then wrapped around the nerve ends, creating a firm connection. The superior laryngeal nerve (SLN) provides collateral innervation to the endolaryngeal musculature in both rats and humans. These collateral innervations may interfere with RLN reinnervation, confounding this and future investigations. To prevent interference, the SLN was exposed by retraction of the omohyoid, ligated proximally and distally using small vessel clips (Ethicon, Somerville, NJ), and then transected between the clips. A 0° 4-mm endoscope (Karl Storz, Tutlingen, Germany) fitted with an epiglottis retractor was inserted transorally to visualize the larynx and confirm right vocal fold paralysis.

Four, 8, 12, 16, and 20 weeks following initial surgery, animals were sedated as described above, transoral laryngeal electromyography (EMG) was performed, and vocal fold motion was evaluated as detailed below and previously. Rats were then sacrificed via an overdose of isoflurane. RLN nerve sections were then harvested proximal and distal to the site of transection. A corresponding section of the left RLN was harvested as a control in each rat. Nerves were subsequently processed as described previously and below. Ultimately, EMG, histologic, and kinesiologic findings were corroborated to evaluate the chronologic evolution of reinnervation and the time needed to allow for maturation of reinnervation.

Kinesology

A 0° 4-mm endoscope (Karl Storz) fitted with an epiglottis retractor was inserted transorally to visualize the larynx. A color LCD camera (Richard Wolf, ) was attached to the endoscope and its signal routed to a color video monitor that was within view of all investigators. Bilateral vocal fold motion was visualized and graded on a scale of 0 to 4, 0 being absence of movement, 1 consistent but minimal motion, 2 purposeful motion with significant paresis, 3 purposeful motion with minimal paresis, and 4 being normal motion compared with the contralateral vocal fold used as a control. Abduction reliably occurs during phasic ventilation, cycling every 600 to 800 ms. Adduction spontaneously occurs during swallow, cough, and reflexive adduction. The scoring was performed by three separate observers. The mode score of observers was used as a final score.

Transoral Electromyography

Animals were placed supine on a modified stereotactic operating table in a Faraday cage recording room. The tongue was retracted using a 3-0 silk suture placed midline in the anterior tongue and suspended. A 0° 4-mm endoscope (Karl Storz) fitted with an epiglottis retractor was inserted transorally to visualize the larynx. Transoral EMG was performed (Figs. 1 and 2). EMG of the left thyroarytenoid and lateral cricoarytenoid muscles (TA/LCA) was undertaken to confirm the absence of motor unit action potentials (MUAPs) during ventilation and its presence during adduction. EMG of the left posterior larynx.

Fig. 1. Transoral electromyography (EMG) using a 0° operating endoscope (A) and an artist rendition of the procedure (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2. Electromyography (EMG) electrode insertion points for measuring consistent strong signals from the posterior cricoarytenoid (red) and thyroarytenoid (green) muscles of the rat larynx. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Cricoarytenoid muscle (PCA) was then performed to confirm the presence of MUAPs during phasic ventilation and absence during adduction. An identical procedure was used for evaluation of the right TA/LCA and PCA. Synkinesis was considered present when consistent MUAPs were observed in the TA/LCA during abduction and in the PCA during adduction. Myoelectric signals were detected using a 25-gauge 45-mm quadrifilar needle electrode (Delsys, Boston, MA). The quadrifilar electrode has four 50-μm-diameter selective surfaces located along cannula side ports that are spaced 200 μm apart in a square pattern. Myoelectric activity was monitored visually using a digital real-time oscilloscope (Tektronix TDS210) and audibly by means of an amplifier/speaker unit connected in parallel to the EMG data recording system. Bipolar EMG signals were recorded from two of the four electrode detectors. Signals were amplified using a preamplifier situated at the needle head and then fed into high-gain differential amplifiers located nearby (in-house construction). Myoelectric signals were bandpass filtered from 15 Hz to 1,000 Hz (Krohn-Hite Model 3360, Brockton, MA), digitized (Data Translation Model DT2821G, Marlboro, MA), and stored in real time (Dolch Computer Systems Model LPAC-PT, Freemont, CA). MUAPs and EMG signals were analyzed using Matlab software by three separate observers (The Mathworks, Release 2007b, Natick, MA).

**Histology**

Isolated RLNs were harvested and all specimens were immediately immersed in a mixture of 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M pH 7.2 phosphate buffer. They were

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**Fig. 3.** Evolution of electromyography (EMG) signals from weeks 4 (A), 8 (B), 12 (C), 16 (D), and 20 (E) at a 20-millisecond sweep speed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
postfixed for 1 hour in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in graded ethanol, and then embedded in epoxy resin. Thin 1-μm sections were cut with glass knives on a Sorvall ultramicrotome and stained with aqueous 1% toluidine blue. Nerve sections were then evaluated under light microscopy. Qualitative evaluation of axon presence and size as well as myelin thickness was performed by two observers using a 40× objective. All nerve sections were compared to determine the maturation of axon and myelin morphology.

RESULTS

Kinesology

Of the 20 rats in five surgical groups, four rats presented volitional motion of the experimental vocal fold prior to sacrifice. Three of these rats were sacrificed 4 weeks postsurgery. One displayed grade 2 vocal fold motion, meaning purposeful movement with severely impaired velocity and range of motion. Two of these four rats had grade 1 motion, meaning a perceptible and consistent twitching motion in phase with the opposite vocal fold. One of these further displayed a continuous tremor of the right vocal fold. One of the four rats sacrificed at 12 weeks postsurgery displayed grade 3 vocal fold motion, meaning a slight decrease in velocity and range of motion. Only abduction was observed in these animals. All other rats displayed no observable volitional motion of the experimental vocal fold on either abduction or adduction, regardless of the time period.

Transoral Electromyography

In all animals, electric silence was never observed. The left vocal fold, serving as a control, showed normal EMG morphology and absence of synkinesis. On the experimental side, there was a clear evolution of the EMG signal observed from week 4 until week 16 (Fig. 3). At week 4, none of the rats showed electromyographic evidence of synkinesis. MUAPs were consistent with complex recovery potentials and significant polyphasic responses. Synkinesis was first observed 8 weeks following surgery (Fig. 4). EMGs revealed MUAPs with continued polyphasic patterns but with the presence of more discrete and narrow units. At week 12, three of four rats displayed synkinesis in the experimental PCA and TA/LCA. Wide polyphasic MUAPs were still present but narrowed compared with week 8. In addition, occasional MUAPs with normal morphology were observed in these three rats. One 12-week rat displayed synkinesis in the PCA only. MUAPs were seen in the TA/LCA when the control vocal fold adducted. This same animal displayed grade 3 vocal fold motion. The morphology of the MUAPs of this animal was similar to the MUAP morphology of the other 12-week animals. By week 16, MUAPs were narrow and mature. Rare polyphasic potentials were observed in one of four animals. There was also a significant increase in recruitment with multiple MUAPs during all recordings. EMGs at week 20 were similar to week 16 with narrow, mature MUAPs and recruitment of multiple MUAPs. Polyphasic MUAPs were not observed in any of the rats at 20 weeks.

Histology

Axon and myelin sheath regeneration plateaued at 16 weeks (Fig. 5). At 4 weeks, there was a significant loss of axons and myelin degeneration with evidence of
Early regeneration as exhibited by thinly myelinated axons of small diameter. The process of myelination occurred quickly. Minimal myelin density changes were observed after week 8. In contrast, as reinnervation progressed, axons increased significantly in diameter. At 16 and 20 weeks, axons resembled the morphologic appearance of the normal control nerve with varying diameter axons. Minimal differences were observed between nerves at weeks 16 and 20. Similar changes occurred in the RLN proximal and distal to the nerve transection.

**DISCUSSION**

Recurrent laryngeal nerve injury occurs at a relatively steady rate during cervical and cardiothoracic surgery. Permanent unilateral vocal fold paralysis occurs in approximately 2% of thyroidectomies, carotid endarterectomies, and open-heart surgeries. The percentages of paralysis in thoracic and skull base surgeries are much higher. Our current treatment methods, while quite helpful, are suboptimal. Optimal treatment of a recurrent laryngeal nerve injury should result in restoration of patient’s dynamic preinjury voice, swallowing, and breathing. Because of the complexity of vocal fold motion and mass control needed to achieve normal laryngeal function, mechanical correction of paralysis or synkinesis reinnervation is unlikely to result in normal functional recovery. Such recovery can only occur if RLN injury and reinnervation can be artificially influenced to occur in an ordered fashion that results in establishment of preinjury innervation patterns. To this end, much research has been focused on investigating the influence of neurotrophic factors on reinnervation and vocal fold function. An appropriate and well-described animal model is integral to conducting this research. The rat is one mammal of choice due to its relatively low cost, simplicity of care, ease of surgery, and high tolerance to intervention. Relatively few investigations have been performed to analyze the natural history of the rat RLN after injury. Because so little is known about recovery after this injury, it is difficult to draw valid conclusions about the effect of interventions, such as the application of neurotrophic factors or antibodies, on the injured nerve.

With the aim of improving our knowledge of RLN injury, this investigation was performed to elucidate the natural evolution of rat RLN transection and anastomosis via evaluation of EMG, kinesiologic, and histologic observations at 4, 8, 12, 16, and 20 weeks posttransection and anastomosis. In addition, the time point at which reinnervation matures was identified to optimize future experimental procedures by minimizing risk of early animal sacrifice, reducing false-negative results, and limiting cost of prolonged animal maintenance.

In the current report, vocal fold motion was absent on the experimental side in 16 of 20 animals following transection. At 4 weeks, three of four animals displayed paretic vocal fold motion. In addition, at 12 weeks, one of four animals in this group displayed grade 3 abduction, no adduction, and synkinesis in the PCA with an absence of synkinesis in the TA/LCA. This phenomenon adds credence to the hypothesis of preferential reinnervation as suggested by Woodson in the cat. One may surmise that contrary to the cat, a rat will preferentially reinnervate its PCA musculature because the primary function of the rat larynx is respiration as opposed to phonation. In addition, while inconclusive, it appears that more rat laryngeal motor neurons innervate the PCA than the TA/LCA, whereas the reverse is likely true in the cat. As such in the rat, preferential PCA reinnervation would be expected. Preferential PCA innervation was observed in the 4-week rats as the PCA was reinnervated predominately by abductor LMNs as evidenced by a lack of EMG synkinesis in the PCA. In contrast, minimal reinnervation of any kind was noted in the TA/LCA. This early innervation pattern resulted in PCA abduction, without antagonistic TA/LCA...
contractions. As reinnervation progressed beyond four weeks, TA/LCA innervation increased. In addition, aberrant mixed reinnervation of both the TA/LCA and PCA muscles became evident. This synkinetic innervation pattern resulted in antagonistic adducting and abducting muscle forces that produced an immobile vocal fold. The evolution of EMG signals during this study supports this hypothesis as all rats after 4 weeks evidenced significant EMG synkinetic reinnervation of the TA/LCA and PCA as well as an immobile vocal fold. However, one 12-week animal showed near normal motion on abduction with an absence of adduction. On adduction, EMG showed normal TA/LCA MUAPs in addition to synkinetic PCA MUAPs. This electromyographic activity pattern results in antagonistic muscle activity on attempted adduction, as both the PCA and TA/LCA are stimulated with the resulting lack of vocal fold. In contrast, on abduction the PCA evidenced normal MUAPs and the TA/LCA was silent. Due to the lack of antagonistic muscle activity, grade 3 abduction occurred. These findings support the hypothesis of preferential PCA reinnervation. In addition, they corroborate Flint’s observation of synkinetic reinnervation after RLN transection and anastomosis in a rat as well as Crumley’s clinical observations that vocal fold immobility in humans is due to synkinetic reinnervation, not insufficient reinnervation.\textsuperscript{20,21} This concept of vocal fold immobility following RLN injury is further supported by a recent report highlighting the presence of significant EMG synkinesis as the most accurate indicator of persistent vocal fold immobility after a RLN injury.\textsuperscript{22} To summarize, these observations suggest that during early reinnervation, the axons reaching the PCA are predominately abductors and as a whole outnumber axons reaching the TA/LCA. As a result, vocal fold motion is possible due to PCA abduction that is unopposed by the weakly reinnervated TA/LCA. As reinnervation proceeds beyond the initial phase and more aberrant axons reach the TA/LCA and PCA, the PCA is no longer able to abduct the vocal fold due to antagonistic muscle contraction. What allowed one animal to develop motion at 12 weeks is unclear, but fortuitous reinnervation following RLN transection, though rare, is not unheard of in the human.\textsuperscript{23}

In addition to observations of synkinesis, EMG studies allowed evaluation of the morphologic evolution of MUAPs. The evolution observed in this study correlates well with what is seen following RLN injury clinically in humans and experimentally in rats.\textsuperscript{14,17,24} These EMG findings also correlate well with histologic evolution of nerve recovery. At 4 weeks, early reinnervation results in significant polyphasic MUAPs that may be referred to as complex recovery potentials due to their greater width and more turns than usual polyphasic potentials. Histologically these MUAPs correlate with the immature reinnervation and continuing axonal degradation. Over time the MUAPs began to narrow and turns decreased as the myelin thickened. Between 4 and 8 weeks, the complex reinnervation potentials evolved into the usual morphology of polyphasic MUAPs. At 12 weeks, although polyphasic MUAPs predominate the EMG, the MUAPs have narrowed further and the turns have continued to decrease. Occasionally normal MUAPs were observed. Histologically this is seen as a general increase in myelin thickness and axon diameter. By 16 weeks, histologically the nerve appears mature. Although myelin thickness plateaus at 12 weeks, there is clearly a significant and startling increase in the diameter of many of the axons between 12 and 16 weeks. Correspondingly, at 16 weeks EMG reveals normal MUAPs with occasional giant waves. In one of four rats, only a rare polyphasic potential was observed. Between 16 and 20 weeks, there was minimal evolution in the EMG or histology. Based on these findings, the reinnervation of a rat RLN after transection and anastomosis takes 16 weeks to mature.

CONCLUSIONS

The goal of this study was to evaluate and describe the histologic, electromyographic, kinesologic, and temporal progression of reinnervation after transection and anastomosis of the recurrent laryngeal nerve in a rat model. Findings in this investigation support previous evidence of preferential reinnervation in the larynx of one antagonistic muscle over the other. In the rat, this preference was demonstrated to be for the PCA. Findings also support the hypothesis that vocal fold immobility after RLN injury is due to aberrant reinnervation, not insufficient reinnervation. Histologic and EMG evolution correlate well over the course of reinnervation. Histologic and electromyographic changes plateaued at 16 weeks, suggesting that it takes 16 weeks for maturation of rat RLN reinnervation after transection and anastomosis.

These histologic, electromyographic, kinesologic, and temporal observations should be considered when evaluating the efficacy of various interventions to restore normal vocal fold mobility following RLN injury. Based on the present observations, final outcomes of the intervention should not be measured until 16 weeks after transection and anastomosis so that reinnervation has had a chance to mature.

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