

6-13-2017

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**Notes/Citation Information**
Published in *Journal of Visualized Experiments*, issue 124, e55837, p. 1-7.



**Digital Object Identifier (DOI)**
https://doi.org/10.3791/55837

Video Article

# Subcutaneous Neurotrophin 4 Infusion Using Osmotic Pumps or Direct Muscular Injection Enhances Aging Rat Laryngeal Muscles

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URL: https://www.jove.com/video/55837
DOI: doi:10.3791/55837

Keywords: Developmental Biology, Issue 124, Growth factors, larynx, osmotic pump, muscle, neurotrophin 4 (NTF4), laryngeal injection, rat, aging

Date Published: 6/13/2017

Citation: Andreatta, R.D., Stemple, J.C., Seward, T.S., McMullen, C.A. Subcutaneous Neurotrophin 4 Infusion Using Osmotic Pumps or Direct Muscular Injection Enhances Aging Rat Laryngeal Muscles. *J. Vis. Exp.* (124), e55837, doi:10.3791/55837 (2017).

## Abstract

Laryngeal dysfunction in the elderly is a major cause of disability, from voice disorders to dysphagia and loss of airway protective reflexes. Few, if any, therapies exist that target age-related laryngeal muscle dysfunction. Neurotrophins are involved in muscle innervation and differentiation of neuromuscular junctions (NMJs). It is thought that neurotrophins enhance neuromuscular transmission by increasing neurotransmitter release. The neuromuscular junctions (NMJs) become smaller and less abundant in aging rat laryngeal muscles, with evidence of functional denervation. We explored the effects of NTF4 for future clinical use as a therapeutic to improve function in aging human laryngeal muscles. Here, we provide the detailed protocol for systemic application and direct injection of NTF4 to investigate the ability of aging rat laryngeal muscle to remodel in response to NTF4 application. In this method, rats either received NTF4 either systemically *via* osmotic pump or by direct injection through the vocal folds. Laryngeal muscles were then dissected and used for histological examination of morphology and age-related denervation.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55837/

## Introduction

Laryngeal muscles contract rapidly and consistently, and are vulnerable to the adverse effects of aging. This constant activity is thought to contribute to voice problems or dysphagia observed in persons over 65 years of age [1,2,3,4,5,6,7]. Several molecular and pathophysiologic mechanisms contribute to this age-related dysfunction. These mechanisms can include remodeling of laryngeal mucosa, muscle fiber atrophy or loss, lack of muscle fiber regeneration or atrophy which causes bowing of the vocal folds and inability of glottis closure [8,9,10,11]. There is no proven medical therapy at this time that can completely prevent or rehabilitate these age-related changes in these muscles.

Modulation of the effectiveness of neuromuscular transmission can greatly influence neuromotor performance. The family of neurotrophins include nerve growth factor (NGF), brain derived nerve growth factor (BDNF), neurotrophin 3 (NTF3) and NTF4 [12,13]. Neurotrophins have been shown to modulate synaptic efficacy[1,4]. Hepatocyte growth factor, transforming growth factor beta and fibroblast growth factor have recently been used in humans for the treatment of vocal fold scarring [15-17]. NTF4 also regulates NMJ effectiveness; mice lacking NTF4 show disassembled NMJs [11,18,19]. These studies lead to promising effects of treatment of aging laryngeal muscle disorders and denervation with growth factors.

Direct injection therapeutics to the tissues of the vocal folds are not unprecedented in humans. For example, local injections of botulinum toxin are currently used as an effective treatment for neurological movement disorders that affect the muscles in the larynx, such as spasmodic dysphonia and bilateral recurrent laryngeal nerve paralysis [20,21]. Hyaluronic acid hydrogel is another injectable, which is used to treat vocal fold scaring and glottal insufficiency [22,23]. Injection laryngoplasty can be used to treat a variety of communication disorders [24]. These direct injection methods hold promise to improve vocal function and swallowing in aging populations.

## Protocol

Use male Fischer 344-Brown Norway rats at 6 and 30 months of age for this protocol. Rats were obtained from the National Institute of Aging rodent colony. We used rats for this study because the structure of the rat larynx is similar to that of the human, functionally serving for airway protection and species-specific vocalizations This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky.

## 1. Anesthesia of Rats

1. Prepare the anesthetics by mixing ketamine hydrochloride (dissociative anesthetic) and xylazine hydrochloride (sedative and analgesic) in buffered saline. The concentrations of ketamine and xylazine in the final solution are 100 mg/8 mg per kg body weight respectively.
2. Inject the anesthetics into the rat by intraperitoneal administration using a syringe with a 25 G needle.
3. Determine that the rat is sufficiently anesthetized by pinching the toe or foot with forceps. If the rat does not react to the pinch, then surgery can begin. If the rat reacts to the toe pinch with reflex or muscle contractions, then wait 1-2 min and repeat the pinch test. If the rat reacts again, replace the rat with a new animal and repeat procedure beginning from step 1.2.
4. Apply ophthalmic ointment to the rat's eyes, after the rat is immobile, to prevent the corneas from drying out.

## 2. Osmotic Pump Implantation

1. Place the rat ventral on the aseptic surgical area. Administer meloxicam as a preanesthetic medication. Administer intraperitoneally at a dosage of 1-4 mg/kg of body weight using a syringe with a 25 G needle.
2. Use clippers to remove an approximately 1" x 1" square of fur from back of neck, and approximately 1" caudal of space between shoulders. Shave as close to the skin as possible.
3. Wet the back and neck with disinfecting ethanol (70%).
4. After shaving, scrub the dorsal aspect of the neck with 3 scrubs in succession of iodine-alcohol finishing with alcohol.
5. Maintain the body temperature of the rat by placing it on a heating pad set to 34 °C.
6. **Fill aseptically prepared osmotic pumps with either 50 µL of NTF4 or saline for systemic NTF4 treatment (Figure 1).**
    1. Use a scalpel to make a horizontal incision approximately 2 cm wide through the skin, just cranial to the space between the scapulae. Lift the posterior edge of the incision with forceps with one hand while inserting the tip of hemostats and gently pushing posterior to the incision.
    2. After the tip of the hemostats is approximately 2 cm cranial to the incision, open the handles on the hemostats, expanding the tips to form a hollow "pocket" subcutaneous to the incision site. This will be the placement site for the pump.
7. Orient the pump delivery portal end first upon insertion to minimize any interaction of the NTF4 and the healing of the pocket incision site.
8. Deliver 50 µL of NTF4 saline for either 7 14 days. The 7 day group received 6.72 mg/day of NTF4 for a total dose of 47.04 mg. The 14-day group received 6.72 mg/day for a total dose of 94.08 mg of NTF4[25].
9. Use 5-0 nylon suture thread, hemostats and forceps to close the incision made for pump placement.
10. Observe the rats for a minimum of 30 min as they recover from anesthesia. Criteria for completion of monitoring include the animal becoming active, moving about the cage, drinking water, and beginning other normal activities such as grooming.
11. Monitor animals daily for the first week by observing the healing of surgical site, normal feed and water consumption and passing of urine/feces, and any abnormal behavioral signs of stress, pain, or other post-operative complications.
12. If the rat appears to be in pain or distress, provide the rat with a 5 mg/kg subcutaneous injection of carprofen once every 24 h for up to 5 days to relieve pain.
13. If there appears to be an infection, consult a veterinarian to ensue that the wound heals properly.
14. Depending on which experimental group the rat in in, remove the 5-0 nylon suture 7-10 days following surgery to prevent irritation from the thread.

## 3. Anesthesia of Rats for Direct Injection

1. Withhold food from the rats the night before the procedure. This ensures that there is no food to block the endoscope or injection needle.
2. Weigh rats and prepare acepromazine 1-2 mg/kg body weight. Inject intramuscularly (the IM location is the left thyroarytenoid muscle).
3. Place the rat in the induction box. Induce anesthesia in the induction box with 5% isoflurane and 1 L $O_2$.
4. Move the rat to a nose cone with 2% isoflurane and 600 mL $O_2$.
5. Determine that the rat is sufficiently anesthetized by pinching the toe or foot with forceps. If the rat does not react to the pinch, then the injection protocol can begin. If the rat reacts to the toe pinch with reflex or muscle contractions, then wait 1-2 min and repeat the pinch test. If the rat reacts again, replace the rat with a new animal and repeat procedure beginning from step 3.4.

## 4. Direct Injection and Visualization

1. Place aseptically prepared 50-µL dosages containing NTF4 or saline in a $H_2O$ bath set to 25° C for 30 min before injection.
2. Place the rat in a supine and reclined position on a plexiglass platform (**Figure 2**). Suspend the rat in the reclined posture from their frontal top incisors via a guide wire strung across the top of the platform.
3. Attach a 50 mm, 30 gauge, 100 µL syringe to a 1.9 mm, 30° sinus endoscope (**Figure 3**).
   NOTE: The syringe assembly is attached via a jig that holds the cannula firmly to the outer wall of the endoscope. The endoscope allows for visualization of the vocal folds and guidance of the syringe intraorally. The position of the cannula tip is adjusted prior to each animal to ensure that the tip is fully and clearly visible via the endoscopic view (**Figure 4**).
4. Use a rubber-tipped pair of forceps to extend the tongue and move it laterally. Afterward, insert a plastic speculum to maintain oral patency. Make the speculum from a 5 mL plastic syringe barrel that is cut to a length of 1.5 to 2 cm, with the cut edges deburred and polished smooth.
5. Turn off the lights in the room and attach a halogen light source to the endoscope. Turn on the video recorder to capture the procedure.
6. Immerse the distal end of the endoscope in warm water for a few seconds to minimize the development of condensation on the glass tip when inserted into the mouth of the rat.
7. Using visual feedback from the monitor, carefully guide the needle to the area of the left vocal fold.
8. **Time the injection of the solution with the inspiratory phase of the animals' respiration cycle to fully access the vocal fold. During the inspiratory phase of respiration, the vocal fold is fully exposed.**

1. Once the vocal fold is fully visible, insert the needle into the left thyroarytenoid, found lateral to the white medial edge of the vocal fold. With the needle in place, deliver the injectate through depression of the syringe.

9. Turn off the halogen light source on the endoscope and the video player, and turn back on the room lights.
10. Return the rat to its home cage and place on a heating pad.
11. Allow the rat to recover before removal from the heating pad. Replace food and water in the cage.
12. Monitor rats for 7 days after the injection and then euthanize. Remove the larynges for cryosectioning[24].

## 5. Euthanization of Rats

1. Anesthetize rats with ketamine hydrochloride and xylazine hydrochloride (100 mg/8 mg per kg body weight injected intraperitoneal injection).
2. Euthanize by exsanguination following a medial thoracotomy.

## Representative Results

The rats were euthanized after 2 weeks of osmotic pump infusion or 1 week after direct injection of NTF4. Larynges were harvested, placed in cryoprotectant (30% sucrose and 70% phosphate buffered saline) and then serially sectioned in 10- µm widths with a cryostat. Aging laryngeal muscles are affected by administration of NTF4 [25]. In addition to young and old rat, we compared the injected and non-injected side of the thyroarytenoid muscles. Typically we see a change in fiber size with age, which varies based on the route of administration of NTF4 (**Figure 5**). Less fibrosis is also qualitatively observed after treatment. The percent of denervated fibers decreases with systemic and direct application of NTF4 in aged rats (**Figure 6**). The quantity of NMJs also increases (**Figure 7**). The significance of this increase depends on the length of treatment or route of administration.

**Figure 1: Representative Image of Osmotic Pump.** After filling the pump with NTF4, the flow moderator is inserted into the main body to seal the pump.

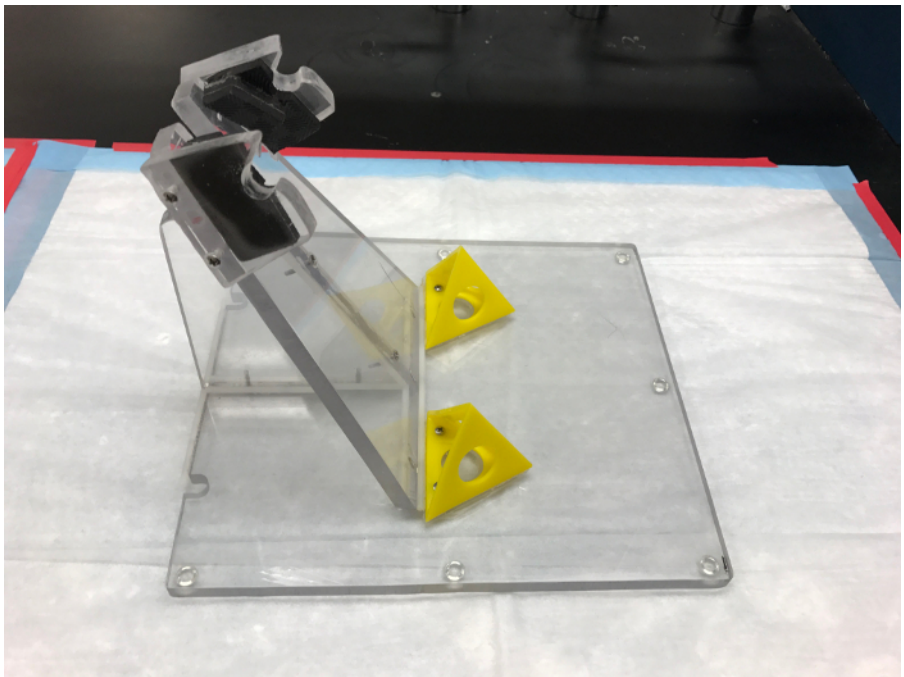

**Figure 2: Platform for Injection Procedure.** The rat is placed in a supine posture on the platform and suspended by the frontal upper incisors via a guide wire mounted to the top of the platform. Please click here to view a larger version of this figure.

**Figure 3: Representative Image of Syringe.** Fill the syringe with NTF4 and saline mixture. Please click here to view a larger version of this figure.

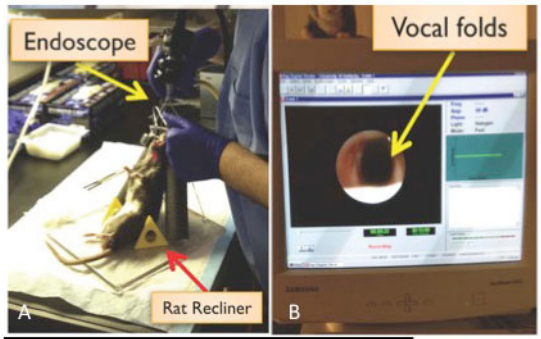


**Figure 4: Injection Procedure.** (**A**) The syringe is coupled to a 19.9 mm 30° endoscope (shown); (B) The optic light is checked to make sure there is light for the procedure; (**C**) The real-time video ensures that the needle is guided to the right location.

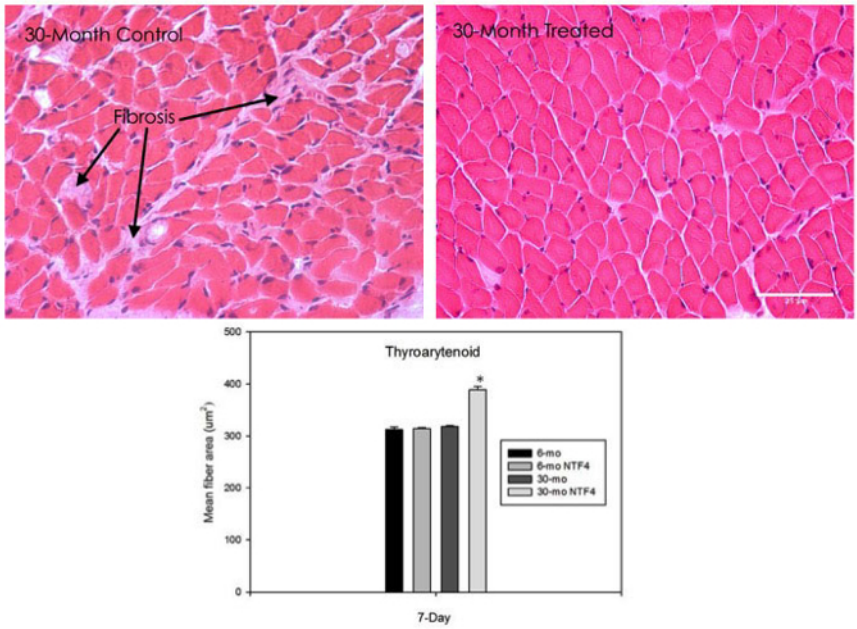


**Figure 5: Changes in Fiber Size and Morphology.** Representative hematoxylin and eosin stained sections of systemic NTF4 7-day treated (right) and untreated (left) thyroarytenoid muscles at 30 month of age[26,27,28]. There is an increase in fiber size from control to treated animals. Treatment with NTF4 changes the 30-mo fiber size to that of a younger 6 month old animal. *p <0.05 versus 30 month (pictures were captured at 40X magnification; Scale bar = 25 µm, P <0.001). Arrows in the top picture point to an area of fibrosis. There is also a qualitative decrease of fibrosis in the treated animals. Please click here to view a larger version of this figure.

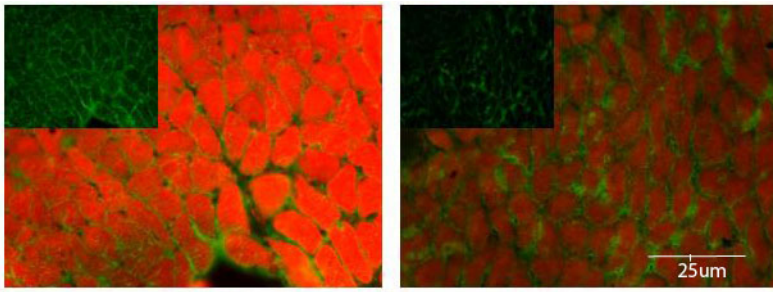


**Figure 6: Changes in Innervations with NTF4 Treatment.** Representative Nav1.5 stained sections from systemic NTF4 14-day treated (right) and untreated (left) thyroarytenoid muscles at 30 month of age (pictures were captured at 40X magnification; Scale bar = 25 µm)[29]. There is a reduction of denervation with age by $Na_v1.5$ labeling (green). Left panel is representative $Na_v1.5$ (green) and phalloidin, to denote fibers (red) stained sections from untreated thyroarytenoid muscles, the right panel is treated muscles. Green insert is $Na_v1.5$ staining alone.

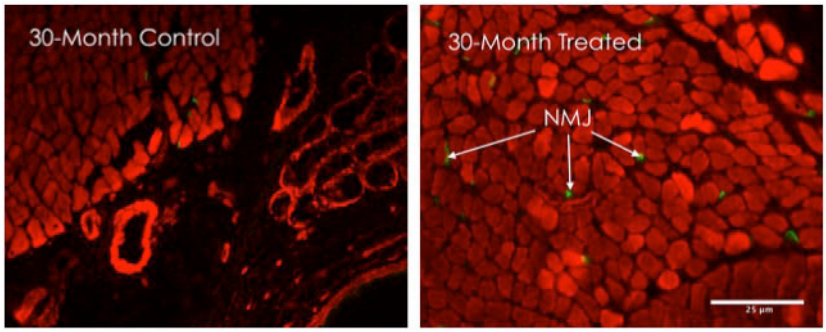


**Figure 7: Changes in NMJs with NTF4 Treatment.** Representative fluorescence microscopy images of NMJs from systemic NTF4 14-day treated thyroarytenoid muscle sections from different animals labeled with α-bungarotoxin (green) and phalloidin (red) showing that NMJ number increases with NTF4 treatment at 30 month (Scale bar = 25 µm), (P <0.001) [11].

## Discussion

Laryngeal muscles are vulnerable to the unfavorable effects of aging. Previous studies have demonstrated changes in aging laryngeal muscles that include changes in fiber size, total number of fibers, regenerative ability, NMJ size and quantity changes, in addition to variations in contractile function and myosin isoform shifts [4,11,27,30,31]. Aging laryngeal muscle can be altered by application of neurotrophins. These changes can be readily measured. Therefore, rat laryngeal muscles provide a useful model to study the effects of aging, and vocal diseases/disorders. The study of these muscles may also help develop interventions or protective processes in the aging human.

Our method of systemic or direct application of NTF4 produces a remodeling response in aging rat laryngeal muscles. This demonstrates that neurotrophins may have therapeutic potential on aging-related muscle dysfunction. The direct application of neurotrophins can easily be translated to the human model of vocal ageing though injection vectors[25].

There are many factors to consider when using the injection protocol. First, care must be taken to prevent scratching and injury to soft tissue around the laryngeal vestibule considering the tip of the endoscope has a sharp needle attached. Second, timing the movement of the needle tip into the larynx during the inspiratory phase of the animals' breath cycle takes practice to accurately time the injection. Third, ensure that the needle tip is clearly visible before attempting the procedure by visually inspecting the apparatus on the computer monitor beforehand. Fourth, warming the tip of the endoscope in hot water for a few seconds is an important step to make certain that fogging of the endoscope tip is avoided. Finally, it is also important to fast the rat the night before any scoping procedure. If food is not withheld it is highly likely that they will still have food residue in the pharynx, which makes the injection procedure virtually impossible to perform. Rats should be inspected for signs of dehydration daily and weighed to make sure there is no significant weight loss (greater that 10% of body weight).

Two critical steps in the development of the protocol were the attachment of the syringe to the endoscope, and the use of an oral speculum. Firm anchoring of the syringe assembly was necessary to facilitate single-handed operation for scope insertion and injection. Considering the goals of the study, in the absence of direct visualization to guide injection of the vocal fold, the experiment could not have been realized. Additionally, the creation of an oral speculum was deemed important to prevent the tongue from moving and to maintain the epiglottis open throughout the procedure.

The visually guided injection method under went several rounds of modification mostly related to finding the optimal means of securely anchoring the syringe and needle to the endoscope. After testing several different forms of tape adhesives, it was found that commercially available elastic athletic tape was the best means of anchoring the syringe to the endoscope body.

The greatest limitation of the visually guided injection method was not related to instrumentation, but rather to the need of having a patent pharynx and airway. Although, restricting food for 24 hours prior to the procedure solved most of this issue, rats will ingest anything in their cages, including bedding and feces. When this occurred, there were two solutions: (1) postpone the injection until the animal clears their pharynx naturally, or (2) attempt to remove the blockage manually using forceps. It was our experience, that the former was the better option because it reduced the risk of potential injury to the animal's pharyngeal region.

Given the novelty of this protocol and the need to directly inject the vocal fold with the compound, no other reliable methods exist. Given the small size of the animals laryngeal system, visually guided injection through the oral and pharyngeal region in the only way to ensure correct and consistent placement of the compound in the living animal. The only other means of directly injecting the vocal fold was to attempt to do so from an external position through the skin and cartilage of the larynx. Although this method is successfully performed in the humans undergoing botulinum toxin therapies to alleviate laryngeal dystonias, transcutaneous injection methods are not feasible in small animals.

The technique is robust and can be used for not only injection of the vocal fold, but for injection sites within the pharyngeal and oral regions. Additionally, the method can be adapted for simple visual monitoring of the animals pharyngeal and laryngeal region by removing the syringe.

In summary, this injection method is a novel means to study biological mechanisms related to the treatment of aging-related voice dysfunctions in humans. This method also has the potential to be applied to other disease models that affect voicing, vocal function, communication, and swallowing in humans.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

This work was supported by grants from the National Institute on Deafness and Other communication Disorders (R21DC010806 to C.A.M. and J.C.S and R01DC011285 to C.A.M.).

## References

1. Trupe, E.H., *et al.* Correlates and consequences of eating dependency in institutionalized elderly. *J. Am. Geriatrics Society.* **34** (3) 192-198 (1986).
2. Ward, P.H., Colton, R., McConnell, F., Malmgren, L., Kashima, H., Woodson, G. Aging of the voice and swallowing. *Otolaryngol. Head Neck Surg.* **100** (4), 283-286 (1989).

3. Gay, E.G., Kronenfeld, J.J., Dettmann, F.G. A comparison on the effect of regulation on health care for the older American: a tale of two states. *Gerontologist.* **34** (6), 787-796 (1994).
4. Hagen, P., Lyons, G.D., Nuss, D.W. Dysphonia in the elderly:diagnosis and management of age-related voice changes. *South. Med. J.* **89** (2), 204-207 (1996).
5. Broniatowski, M., *et al.* Current evaluation and treatment of patients with swallowing disorders. *Otolaryngol. Head Neck Surgery.* **120** (4), 464-473 (1999).
6. Lundy, D., S., *et al.* Aspiration: cause and implications. *Otolaryngol. Head Neck Surg.* **120** (4), 474-478 (1999).
7. Schindler, J.S. and Kelly, J.H. Swallowing disorders in the elderly. *Laryngoscope.* **112** (4), 589-602 (2002).
8. Gambino, D.R., Malmgren, L.T., Gacek, R.R. Age-related changes in the neuromuscular junctions in the human posterior cricoarytenoid muscles: a quantitative study. *Laryngoscope.* **100** (3), 262-268 (1990).
9. Sinard, R.J. The aging voice: how to differentiate disease from normal changes. *Geriatrics.* **53** (7), 76-79 (1998).
10. Baker, K.K., Olson-Raming, L., Sapir, S., Luschei, E.S., Smith, M.E. Control of vocal loudness in young and old adults. *J. Speech Lang. Hear Res.* **44** (2), 297-305 (2001).
11. McMullen, C.A. and Andrade, F.H. Functional and morphological evidence of age-related denervation in rat laryngeal muscles. *J. Gerontol. A. Biol. Sci. Med. Sci.* **64** (4) 435-442 (2009).
12. Lai, K. and Ip, N.Y. Postsynaptic signaling of new players at the neuromuscular junction. *J. Neurocytol.* **32** (5-8), 727-741 (2003).
13. Huang, E.J. and Reichardt, L.F. Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24** (1) 677-736 (2012).
14. Gonzalez, M., *et al.* Disruption of Trkb mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. *Neuron.* **24** (3), 567-583 (1999).
15. Hirano, S., Kishimoto, Y., Suehiro, A., Kanemaru, S., Ito, J. Regeneration of aged vocal folds: first human case treated with fibroblast growth factor. *Laryngoscope.* **118** (12), 2254-2259 (2008).
16. Branski, R.C., *et al.* Effects of transforming growth factor-beta 1 on human vocal fold fibroblasts. *Ann. Oto. Rhinol. Laryngol.* **118** (3), 218-226 (2009).
17. Kishimoto, Y., *et al.* Effects of exogenous hepatocyte growth factor on vocal fold fibroblasts. *Ann. Otol. Rhinol. Laryngol.* **118** (8), 606-611 (2009).
18. Belluardo, N., *et al.* Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. *Mol. Cell. Neurosci.* **18** (1), 56-57 (2001).
19. Johnson, A.M., Ciucci, M.R., Connor, N.P. Vocal training mitigates age-related changes within the vocal mechanisms in the old rat. *J. Gerontol. A Biol. Sci. Med. Sci.* **68** (12), 1458-1468 (2013).
20. Roscow, D.E., Parikh, P., Vivero, R.J., Casiano, R.R., Lundy, D.S. Considerations for initial dosing of botulinum toxin in treatment of adductor spasmodic dysphonia. *Oto. HeadNeck Surg.* **148** (6), 1003-1006 (2013).
21. Benninger, M.S., Hanick, A., Hicks, D.M. Cricothyroid muscle botulinum toxin injection to improve airway for bilateral recurrent laryngeal nerve paralysis, A case series. *J. Voice.* **30** (1), 96-99 (2016).
22. Coppoolse, J.M.S., *et al.* An in vivo study of composite microgels based on hyaluronic acid and gelatin for the reconstruction of surgically injured rat vocal folds. *J. Speech Lang Hear Res.* **57** (2), S658-S673 (2014).
23. Hertegård, S., *et al.* Cross-linked hyaluronan used as augmentation substance for treatment of glottal insufficiency: Safety aspects and vocal fold function. *Laryngoscope.* **112** (12), 2211-2219 (2002).
24. Walker, W.F. Jr., and Homberger, D.G. *Anatomy and dissection of the rat.* Third Edition. W.H. Freeman and Company, New York, (1997).
25. Ohno, T., Hirano, S., Rousseau, B. Age-associated changes in the expression and deposition of vocal fold collagen and hyaluronan. *Ann. Oto. Rhinol. Laryngol.* **25** (1), 192-197 (2009).
26. Stemple, J.C., *et al.* Enhancement of aging rat laryngeal muscles with endogenous growth factor treatment. *Physiol. Rep.* **4** (10), e12798 (2016).
27. McMullen, C.A. and Andrade, F.H. Contractile dysfunction and altered metabolic profile of the aging rat thyroarytenoid muscle. *J. Appl. Phys.* **100** (2), 602-608 (2006).
28. Engle, W.K. and Cunningham, G.C. Rapid examination of muscle tissue. An improved trichrome method for fresh-frozen biopsy section. *Neurology.* **Nov** (13), 919-923, (1963).
29. Sheehan, D.C. and Hrapchack, B.B. *Theory and practice of histotechnology.* Battelle. Columbus, OH. (1980).
30. Kulakowski, S.A., Parker, S. D., Personius, K.E. Reduced TrkB expression results in precocious age-like changes in neuromuscular structure, neurotransmission, and muscle function. *J. Appl. Phys.* **111** (3), 844-852 (2011).
31. Nishida, N., *et al.* Age-related change in rat intrinsic laryngeal muscles: analysis of muscle fibers, muscle fiber proteins and subneural apparatuses. *Eur Arch Otorhinolaryngol.* **270** (3), 975-984 (2013).


 