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Differences in Neuromuscular Junctions of Laryngeal and Limb Muscles in Rats

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Abstract

Objectives/Hypothesis—Laryngeal muscles are specialized for fine control of voice, speech and swallowing, and may differ from limb muscles in many aspects. Since muscles and their controlling motor neurons communicate via neuromuscular junctions (NMJ), we hypothesized that NMJs in laryngeal muscles have specialized characteristics different from limb muscles.

Study Design—In vivo study.

Methods—Single muscle fibers from 12 Sprague-Dawley rats (6 male, 6 female) were used to analyze the postsynaptic side of NMJs from laryngeal thyroarytenoid (TA), cricothyroid (CT), posterior cricoarytenoid (PCA), limb soleus (SOL), and extensor digitorum longus (EDL) muscles. NMJs were labeled with rhodamine-conjugated α -bungarotoxin. With confocal microscopy, we counted cluster fragments, and measured the NMJ area, both absolute and normalized (corrected by muscle fiber diameter) for at least 10 single fibers from each muscle of each animal. Differences between genders were also compared.

Results—Cluster fragments of postsynaptic NMJs were more numerous in PCA and TA compared to CT, SOL and EDL muscles ($P < .01$) in both male and female rats. NMJ cluster fragments were more numerous in female than in male rats only in the TA muscle ($P < .01$). The absolute area covered by the NMJs showed SOL > EDL > PCA > CT > TA ($P < .01$), however, with normalization, the SOL = EDL = PCA > CT = TA.

Conclusions—Differences found in NMJ surface and organization between laryngeal and limb muscle fibers may relate to specialized laryngeal muscle functions. Differences in NMJs between male and female rats were found only in the TA muscle, suggesting an underlying mechanism for some gender-specific laryngeal disorders related to abnormal TA muscle activity.

Level of Evidence—Outcomes research

Keywords

Laryngeal muscle; neuromuscular junction; single fiber; cluster fragments; gender difference; rat

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INTRODUCTION

The intrinsic laryngeal muscles control the shape and tension of the vocal folds which mediate fine control of phonation, vocalization, respiration, swallowing and airway protection in mammals. Laryngeal muscles have rapid shortening speed, high active tension and high fatigue resistance¹⁻³. Muscle activity is directly modulated by nerve impulses from motor neurons via axons to muscle fibers to induce muscle contractions. The junction between a motor neuron and a muscle fiber is referred to as the neuromuscular junction (NMJ). Postsynaptic components of NMJs include acetylcholine receptors (AChRs) which accept acetylcholine released from the nerves, thus evoking a muscle impulse. Each adult single muscle fiber maintains an NMJ of the specific appropriate size, efficiency and spatial distribution to maintain its functional properties.

Important functional and structural differences exist between limb and cranial muscles. For instance, cranial muscles have higher ratios of end-plate size to muscle fiber volume than the limb muscles⁴. In addition, intrinsic laryngeal muscles were recently reported to be spared or less affected in some degenerative neuromuscular diseases⁵⁻⁹ with increased fragmentation of NMJs. Considering that fine structure of the NMJ is closely related to its function⁴, the specialized functional regulation of the laryngeal muscles may be due to their specific NMJ morphology. Such differences may, in turn, make it less likely for neuromuscular diseases to injure laryngeal muscles.

Comparing the NMJs of limb and laryngeal muscles could increase our understanding of the mechanisms underlying these reported differences. Fine structural comparisons between limb and cranial muscle NMJs are lacking. Most studies have focused on either limb or laryngeal muscles with an emphasis on the effect of aging and degenerative disorders. In addition, most of these studies have investigated morphological changes in the NMJ with aging in whole laryngeal muscles^{10,11}. A more detailed structural comparison between limb and cranial muscle NMJs is therefore needed.

Recent evidence suggests a possible difference in laryngeal muscle NMJ structure and function between genders. For example, females are much more likely (~80% predominance) to be affected by spasmodic dysphonia (SD)¹²⁻¹⁴, a disease characterized by the involuntary movements and inconsistent contractions of the intrinsic laryngeal muscles. Intramuscular botulinum toxin injections are used to inhibit acetylcholine release¹⁵ from nerve endings and to decrease the amount of ACh binding to AChR, therefore reducing muscle contractions. Some reports indicate that the amount of botulinum toxin required to treat SD may be lower in men, and that the side effects of breathiness (loss of voice) due to denervation are greater in men¹⁶. The mechanisms underlying these gender differences in prevalence and treatment are rarely studied and remain unknown. Possible NMJ structural differences in laryngeal muscles between genders could be one of many underlying mechanisms.

Most studies of NMJ morphologies and structures are performed on whole muscle tissue sections and use alpha-bungarotoxin, a drug that binds to the AChR and thus labels the postsynaptic site of the NMJ. In the present study, we use the same label but we examined the postsynaptic NMJ morphology of single muscle fibers isolated from laryngeal muscles of the Sprague-Dawley rat. We studied three intrinsic laryngeal muscles, i.e. the posterior cricoarytenoid (PCA), cricothyroid (CT) and thyroarytenoid (TA); and two limb muscles, the slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL). We hypothesized that 1) NMJs of laryngeal muscles would show a different morphology from that of the limb muscles; 2) NMJs would show different morphologies among individual

laryngeal muscles; and, 3) laryngeal NMJ morphology differences may exist between genders.

MATERIALS AND METHODS

Animals

Twelve young adult Sprague-Dawley rats (Harlan, Indianapolis, IN) including 6 males and 6 females, were used for this experiment. All rats were maintained on a 12-hour light/dark cycle and given *ad libitum* access to food and water. All procedures were carried out in accordance with National Institutes of Health guidelines on the care and use of laboratory animals. The study protocol was approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee.

Isolation of laryngeal and limb muscles

Rats were euthanized with an intraperitoneal injection of sodium pentobarbital (190 mg/kg body weight) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by a freshly prepared cold 4% paraformaldehyde (PFA) solution in 0.1 M PBS. Under an operating microscope (Zeiss, LR66238C, Germany), the PCA, CT, TA, SOL and EDL muscles from the right side of each animal were isolated. All the muscles were fixed in 4% PFA at room temperature for two hours and then moved into 0.1 M PBS.

Histological analysis of the postsynaptic NMJ in single muscle fibers

Muscles were longitudinally cut into small pieces and the mid-portion from the same area in each muscle was selected for fiber separation. Single muscle fibers in each muscle were identified, separated apart and stained for acetylcholine receptors (AChR, postsynaptic NMJ) following the procedure described below. Muscle fibers were incubated in 4 % bovine serum albumin (BSA) blocking solution for 1 hour, then moved to tetramethylrhodamine α -bungarotoxin (BTX, 1:500; Invitrogen, CA, USA) diluted in 0.1M PBS for 1 hour at 4°C to label AChR. After 3 washes with 0.1M PBS, muscle fibers were incubated in Hoechst 33342 (1:500, Sigma) for 15 minutes to label the nuclei, then washed in PBS three times, mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA), and sealed.

NMJ images were acquired on a Zeiss LSM 510 confocal microscope with a 40x N.A. 1.4 objective lens as a series of optical sections separated by 1 μ m increments. Series were recorded regardless of the orientation of the NMJ in relation to the microscope focus plane. A three-dimensional (3D) reconstituted image of each labeled postsynaptic NMJ was obtained by doing 64 maximum projections of the data, covering 360 degrees around the Y axis. A 3D movie of each NMJ was created (see supplementary material). The 3D movie was used, for each NMJ, to determine the projection that best reflected the total size of the NMJ. This 2D projection was then used for the measurements. The muscle fiber diameter was determined from the background Hoechst staining. At least 125 images of muscle fibers with AChR staining were taken from each muscle and from 12 animals.

Morphology/size of the postsynaptic NMJs was analyzed using ImageJ software (developed by Wayne Rasband at the National Institutes of Health and freely available at rsbweb.nih.gov). The discontinuous fragments of each postsynaptic NMJ were counted and the muscle fiber area covered by each NMJ was measured. Muscle fiber diameter was measured to calculate the normalized postsynaptic NMJ area (postsynaptic NMJ area/muscle fiber diameter). The largest diameter adjacent to the postsynaptic NMJ on 2D stack images was measured. After developing the measurement methods, a pilot study for the first ten images of NMJs was performed by two independent investigators (XF and TZ) and their

results were compared. As no differences were found, all of the measures were made by one investigator while blinded to the muscle group identity.

Statistical Analysis

The images were analyzed by an investigator blinded to the muscle groups. All values were expressed as the mean $\pm$ standard deviation. Data were analyzed by two-way ANOVA with gender (male, female) and different muscles (PCA, CT, TA, SOL and EDL) as grouping variables. Post-hoc analysis was performed using a *t*-test with the Bonferroni adjustment method. Differences were considered significant at $P < 0.05$.

RESULTS

Morphology of postsynaptic NMJ in different skeletal muscles

Figure 1 shows representative 2D images of NMJs in five different skeletal muscles. The soleus and EDL muscles show the classical appearance of NMJs, sometimes referred to as “pretzel-shaped”. In contrast, AChRs in PCA and TA muscles were grouped into multiple distinct small, rounded clusters, whereas the NMJ in CT muscle looked more similar to the EDL muscle.

Quantification confirmed that in both male and female rats, postsynaptic NMJs of the PCA and TA had more fragments compared to the SOL and EDL muscles ($P < .05$), whereas CT muscle showed similar amounts of cluster fragments compared to the SOL and EDL muscles ($P > .05$) (Figs. 2A, 2B). SOL had more NMJ cluster fragments than the EDL ($P < .01$). No difference was found between PCA and TA muscles ($P > .05$). A significant gender difference was found in the TA muscle, where the females showed more cluster fragments than the males ($P < .01$, Fig. 2C). In the PCA muscle, there was a tendency toward more cluster fragments in the female rats than the male rats; the difference was not statistically significant, which may be due to a larger standard deviation ($P > .05$).

Occasionally, multiple NMJs were observed in a single muscle fiber (Fig. 3). These types of muscle fiber were only found in PCA muscle (3 among 125 fibers from 12 rats) in this study.

Measurement of NMJ surface area in different skeletal muscles

Table I lists the resulting measurements of NMJs from these experiments. Because no gender differences were found in these measures, data from both male and female rats were combined and the comparison was made between different muscles. Limb muscles showed the biggest NMJ area (SOL>EDL>PCA>CT>TA, $P < .05$) and muscle fiber diameter (SOL>EDL>PCA>CT>TA, $P < .05$), but normalized NMJ area was similar to the PCA ($P > .05$) (SOL=EDL=PCA>CT=TA). For all three laryngeal muscles analyzed, PCA showed the biggest normalized NMJ area compared to the CT and TA muscles ($P < .05$).

DISCUSSION

The purpose of this study was to compare the morphology of neuromuscular junctions (NMJs) at the single muscle fiber level in young adult Sprague-Dawley rats in laryngeal and limb muscles. The advantage of studying the NMJ in single muscle fibers is the ability to view the whole NMJ regardless of its orientation and to match the morphology of each NMJ precisely to its muscle fiber. A recently published paper suggested that 3-D measurements are recommended for NMJ morphology testing¹⁷, but measurements of 2-D projections appear ideal to identify the changes of NMJ in morphology and size¹⁵.

Taking advantage of confocal microscopy, we combined the 3-D image collection and 2-D projection so that the full surface area of each NMJ could be measured regardless of its orientation and matched to the fiber diameter accurately. The major findings were: 1) the PCA and TA muscles had more fragmented NMJs than the CT, SOL and EDL muscles; 2) only the TA muscle showed male-female differences (more fragmented NMJ cluster fragments in the female than in the male rats); 3) multiple NMJs were found in some fibers of the PCA muscle; and 4) PCA, SOL and EDL muscles had larger normalized NMJ areas than the TA and CT muscles. In the rat the laryngeal muscles are used for precise patterns of ultrasonic vocalization¹⁸ and in the human for speech, which both require precise muscle tension control. Rats may provide a good model for studying the NMJ structure and distribution in single muscle fibers of laryngeal muscles due to their size and easy accessibility for tissue collection. Large numbers of whole fibers can be collected, ensuring that NMJs will be present in each fiber, and large numbers of animals can be collected, improving the reliability of the results. In addition, the fiber types in the selected limb and laryngeal muscles are well known. For example, among laryngeal muscles in rats, the TA, an adductor muscle, expresses more fast fibers (100%) than PCA (80%), the abductor, and CT (70%), the vocal fold tensor¹⁹. TA and PCA showed more fragments compared to the CT muscle, suggesting that these two muscles may express similar muscle twitch patterns during the modulation of laryngeal adduction and abduction. CT muscle was the most similar in morphology and fragmentation to the EDL muscle, which is composed of 90% fast-twitch fibers. However, the limb muscle of the rat, SOL, a representative muscle with mostly slow-twitch muscle fibers (90%) showed more fragments compared to the EDL. It seems that the limb muscles do not follow the rule of intrinsic laryngeal muscles - the higher the proportion of fast-twitch fibers, the higher the number of NMJ cluster fragments. A possible explanation for these differences is that the shape and size of NMJs could change significantly depending on muscle fiber types and physiological status in a single muscle²⁰⁻²³. NMJ development, maturation and maintenance are determined by the signals between the nerve terminal and the muscle fiber, which play distinct roles in postsynaptic differentiation of the neuromuscular synapse^{24,25}. Specifically, development of intrinsic laryngeal muscles and their innervation differs from that in limb muscles. For example, human laryngeal innervation shows delayed maturation compared to other skeletal muscles²⁶ which may suggest that the development and maturation of laryngeal muscle innervation could be further modulated by their specialized functions after birth.

In addition to the differences between laryngeal and limb muscles, there are also marked differences in NMJ cluster morphology among different intrinsic laryngeal muscles. These differences may underlie the distinct sensitivities of laryngeal muscles to muscle diseases. For example, intrinsic laryngeal muscles are spared from myonecrosis in the *mdx* mouse model of Duchenne muscular dystrophy (AChRs are fragmented in the affected muscles)⁵⁻⁸, except the CT muscle. Interestingly, another neuromuscular disease, myasthenia gravis, also shows more severe electrophysiological pathology in CT muscle compared to other intrinsic laryngeal muscles⁹. The highly specialized morphology of NMJs may predispose some laryngeal muscles to be more sensitive or resistant to some neuromuscular diseases.

However, in some laryngeal diseases with increased muscle tension, like spasmodic dysphonia (SD), the PCA and TA muscles show higher levels of activity²⁷. This may reflect possible correlates of higher synapse strength with the specialized NMJ clusters. In support of this concept, synapse strength of laryngeal muscles is higher in females and can be increased by the female hormone, estrogen^{28,29}. In our study, the TA muscle in female rats had more AChR clusters than in male rats, which strongly suggests a correlation between larger numbers of AChR clusters and higher synapse strength. The PCA muscle also had the largest NMJ area compared to the other intrinsic laryngeal muscles.

Our findings could provide a valuable model for future testing of the possible link between NMJ organization and synapse strength in humans, and might provide useful information for the development of NMJ targeting strategies for treating laryngeal muscle abnormalities. For example, the PCA muscle may play a role in abductor spasmodic dysphonia (ABSD) where botulinum toxin injection has not consistently been therapeutically helpful³⁰. If it were shown that our results could be extrapolated to human PCA NMJs then it might be investigated whether higher numbers of synapses may require higher doses of botulinum toxin for injection.

In addition to the fine structural differences, we found a few single muscle fibers displaying multiple NMJs, consistent with reports of human laryngeal muscles innervated at multiple NMJs by a single axon^{26,31}. We did not stain the neural axon in single muscle fibers in the present study. Perhaps multiple NMJs in rats might be related with vocalization and might be correlated with the fine control of vocal fold movement required for speech in humans, similar to the multi-innervation reported in extraocular muscles³². The similar observations of multiple NMJs in laryngeal muscles suggest homology between rats and humans in laryngeal muscle innervation. However, there are differences between rat and human laryngeal muscle physiology and muscle composition. For example, laryngeal muscles in the rat comprise more fast-twitch fibers than those in humans¹⁷. Therefore, more work is needed to substantiate some of the differences found here in the rat between limb and laryngeal muscles, in humans. The present study did not test the fiber type (fast or slow twitch fiber) or the single muscle fiber function for each NMJ analyzed. Furthermore, human samples are irreplaceable when it comes to the study of speech and voice disorders. The rat model remains valuable, however, for further studies on the development of innervation and of physiological functioning in the laryngeal muscles. Ultimately, linking NMJ morphology to single fiber type/function could help provide a better understanding of laryngeal muscles.

CONCLUSION

Rat intrinsic laryngeal muscles, especially the PCA and TA muscles, present specialized post-synaptic NMJs compared to the limb muscles. Further study of these NMJ functions will improve our understanding of laryngeal muscles specifically affected or spared in neurological disorders. The NMJ gender differences in the TA muscle may suggest an underlying mechanism to examine in future research for the study of gender-related differences in some laryngeal disorders related to abnormal TA muscle activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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BIBLIOGRAPHY

1. Sciote JJ, Morris TJ, Brandon CA, Horton MJ, Rosen C. Unloaded shortening velocity and myosin heavy chain variations in human laryngeal muscle fibers. *Ann Otol Rhinol Laryngol*. 2002; 111:120–127. [PubMed: 11860063]
2. Johns MM, Urbanchek M, Chepeha DB, Kuzon WM Jr, Hogikyan ND. Length-tension relationship of the feline thyroarytenoid muscle. *J Voice*. 2004; 18:285–291. [PubMed: 15331100]
3. Cooper DS, Rice DH. Fatigue resistance of canine vocal fold muscle. *Ann Otol Rhinol Laryngol*. 1990; 99:228–233. [PubMed: 2310139]
4. Pfister J, Zenker W. The Splenius capitis muscle of the rat, architecture and histochemistry, afferent and efferent innervation as compared with that of the quadriceps muscle. *Anat Embryol*. 1984; 169:79–89. [PubMed: 6721223]
5. Marques MJ, Ferretti R, Vomero VU, Minatel E, Neto HS. Intrinsic laryngeal muscles are spared from myonecrosis in the mdx mouse model of Duchenne muscular dystrophy. *Muscle Nerve*. 2007; 35:349–353. [PubMed: 17143878]
6. Thomas LB, Joseph GL, Adkins TD, Andrade FH, Stemple JC. Laryngeal muscles are spared in the dystrophin deficient mdx mouse. *J Speech Lang Hear Res*. 2008; 51:586–595. [PubMed: 18506037]
7. Smythe GM. Dystrophic pathology in the intrinsic and extrinsic laryngeal muscles in the mdx mouse. *J Otolaryngol Head Neck Surg*. 2009; 38:323–336. [PubMed: 19476764]
8. Ferretti R, Neto HS, Marques MJ. Expression of utrophin at dystrophin-deficient neuromuscular synapses of mdx mice: a study of protected and affected muscles. *Anat Rec*. 2011; 294:283–286.
9. Xu W, Han D, Hou L, Hu R, Wang L. Clinical and electrophysiological characteristics of larynx in myasthenia gravis. *Ann Otol Rhinol Laryngol*. 2009; 118:656–661. [PubMed: 19810607]
10. Connor NP, Suzuki T, Lee K, Sewall GK, Heisey DM. Neuromuscular junction changes in aged rat thyroarytenoid muscle. *Ann Otol Rhinol Laryngol*. 2002; 111:579–586. [PubMed: 12126012]
11. McMullen CA, Andrade FH. Functional and morphological evidence of age-related denervation in rat laryngeal muscles. *J Gerontol A Biol Sci Med Sci*. 2009; 64:435–442. [PubMed: 19223602]
12. Adler CH, Edwards BW, Bansberg SF. Female predominance in spasmodic dysphonia. *J Neurol Neurosurg Psychiatry*. 1997; 63:688. [PubMed: 9408119]
13. Asgeirsson H, Jakobsson F, Hjaltason H, Jonsdottir H, Sveinbjornsdottir S. Prevalence study of primary dystonia in Iceland. *Mov Disord*. 2006; 21:293–298. [PubMed: 16211610]
14. Schweinfurth JM, Billante M, Courey MS. Risk factors and demographics in patients with spasmodic dysphonia. *Laryngoscope*. 2002; 112:220–223. [PubMed: 11889373]
15. Suzuki T, Maruyama A, Sugiura T, Machida S, Miyata H. Age-related changes in two- and three-dimensional morphology of type-identified endplates in the rat diaphragm. *J Physiol Sci*. 2009; 59:57–62. [PubMed: 19340562]
16. Ludlow CL, Hallett M, Rhew K, et al. Therapeutic use of type-F botulinum toxin. *New Engl J Med*. 1992; 326:349–350. [PubMed: 1728750]
17. Johnson AM, Connor NP. Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue. *Muscle Nerve*. 2011; 43:203–211. [PubMed: 21254085]
18. Ciucci MR, Ma ST, Fox C, Kane JR, Ramig LO, Schallert T. Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol: A preliminary study. *Behav Brain Res*. 2007; 182:284–289. [PubMed: 17397940]
19. Inagi K, Schultz E, Ford CN. An anatomic study of the rat larynx: Establishing the rat model for neuromuscular function. *Otolaryng Head Neck*. 1998; 118:74–81.
20. Anis NA, Robbins N. General and strain-specific age-changes at mouse limb neuromuscular-junctions. *Neurobiol Aging*. 1987; 8:309–318. [PubMed: 2888031]
21. Santos AF, Caroni P. Assembly, plasticity and selective vulnerability to disease of mouse neuromuscular junctions. *J Neurocytol*. 2003; 32:849–862. [PubMed: 15034272]
22. Prakash YS, Sieck GC. Age-related remodeling of neuromuscular junctions on type-identified diaphragm fibers. *Muscle Nerve*. 1998; 21:887–895. [PubMed: 9626248]

23. Torrejais MM, Soares JC, Matheus SMM, Mello JM, Francia-Farje LAD, Vicente EJD. Morphometric and morphological analysis of neuromuscular junction alterations in the denervated rat diaphragm. *Int J Morphol*. 2009; 27:1235–1242.
24. Belluardo N, Westerblad H, Mudo G, et al. Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. *Mol Cell Neurosci*. 2001; 18:56–67. [PubMed: 11461153]
25. Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci*. 2001; 2:791–805. [PubMed: 11715056]
26. Perie S, St Guily JL, Seville A. Comparison of perinatal and adult multi-innervation in human laryngeal muscle fibers. *Ann Oto Rhinol Laryn*. 1999; 108:683–688.
27. Cyrus CB, Bielamowicz S, Evans FJ, Ludlow CL. Adductor muscle activity abnormalities in abductor spasmodic dysphonia. *Otolaryng Head Neck*. 2001; 124:23–30.
28. Tobias ML, Kelley DB. Sexual differentiation and hormonal regulation of the laryngeal synapse in *Xenopus laevis*. *J Neurobiol*. 1995; 28:515–526. [PubMed: 8592111]
29. Wu KH, Tobias ML, Kelley DB. Estrogen receptor expression in laryngeal muscle in relation to estrogen-dependent increases in synapse strength. *Neuroendocrinology*. 2003; 78:72–80. [PubMed: 12915759]
30. Bielamowicz S, Squire S, Bidus K, Ludlow CL. Assessment of posterior cricoarytenoid botulinum toxin injections in patients with abductor spasmodic dysphonia. *Ann Oto Rhinol Laryn*. 2001; 110:406–412.
31. Perie S, StGuily JL, Callard P, Seville A. Innervation of adult human laryngeal muscle fibers. *J Neurol Sci*. 1997; 149:81–86. [PubMed: 9168170]
32. Zhou Y, Liu D, Kaminski HJ. Pitx2 Regulates Myosin Heavy Chain Isoform Expression and Multi-Innervation in Extraocular Muscle. *J Physiol*. 2011

Fig. 1. Representative confocal images of postsynaptic neuromuscular junctions (NMJ) of single muscle fibers

Postsynaptic NMJ was identified by tetramethylrhodamine α -bungarotoxin (BTX) staining for acetylcholine receptors (AChR) in hindlimb soleus (SOL) and extensor digitorum longus (EDL) muscles of the leg; and in laryngeal cricothyroid (CT), posterior cricoarytenoid (PCA), and thyroarytenoid (TA) muscles. NMJs in the PCA and TA muscle show more fragments. The pattern of postsynaptic NMJ in the CT muscle is similar to that in the limb muscles, especially the EDL. Bar=10 μ m.

Fig. 2. Quantification of AChR cluster fragments in single muscle fibers
 (A) Numbers of NMJ cluster fragments in different muscles in male and female (B) rats. (C)
 Gender differences between NMJ cluster fragments in the same muscle.

Fig. 3. Three NMJs in a single muscle fiber in the PCA muscle of a female rat
Bar=10 μ m.

TABLE I

Neuromuscular junction (NMJ) areas of single muscle fibers in laryngeal and limb muscles.

Muscle	NMJ area (μm^2)	Muscle fiber diameter (μm)	Normalized NMJ area ($\mu\text{m}^2/\mu\text{m}$)
CT	336.07 $\pm$ 12.34	38.04 $\pm$ 1.30	9.25 $\pm$ 0.45
PCA	572.04 $\pm$ 18.52 ^a	42.42 $\pm$ 2.29 ^a	13.99 $\pm$ 0.53 ^a
TA	285.03 $\pm$ 10.07 ^{a,b}	30.68 $\pm$ 1.38 ^{a,b}	9.56 $\pm$ 0.49 ^b
EDL	746.89 $\pm$ 38.50 ^{a,b,c}	56.57 $\pm$ 2.47 ^{a,b,c}	13.42 $\pm$ 0.63 ^{a,c}
SOL	981.27 $\pm$ 59.168 ^{a,b,c,d}	73.52 $\pm$ 2.62 ^{a,b,c,d}	13.61 $\pm$ 0.68 ^{a,c}

Values are means $\pm$ standard deviation

^aSignificant difference ($P < 0.05$) vs. CT;

^bSignificant difference ($P < 0.05$) vs. PCA;

^cSignificant difference ($P < 0.05$) vs. TA;

^dSignificant difference ($P < 0.05$) vs. EDL.

CT, cricothyroid; PCA, posterior cricoarytenoid; TA, thyroarytenoid; EDL, extensor digitorum longus; SOL, soleus.