Research Article

Laryngeal Neuromuscular Response to Short- and Long-Term Vocalization Training in Young Male Rats

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Purpose: Although vocal training is often purported to restore and rebalance laryngeal muscle function, little is known about the direct effects of vocal training on the laryngeal muscles themselves. Consequently, parameters of vocal exercise dose, such as training duration and intensity, have not been well defined. The goal of this study was to use a behavioral animal model to determine the effects of short- and long-term ultrasonic vocalization (USV) training on USV acoustics, thyroarytenoid (TA) muscle neuromuscular junctions (NMJs), and TA muscle fiber size in adult rats.

Method: Twenty-four young adult male Long-Evans rats were divided into 3 groups (untrained control, 4-week training, and 8-week training). Baseline and posttraining USVs were recorded and acoustically analyzed for fundamental

frequency, frequency bandwidth, amplitude, and duration. Presynaptic and postsynaptic NMJ morphological features and muscle fiber size were measured in the TA.

Results: USV training had no effect on USV acoustics. Eight weeks of USV training, however, resulted in a lower NMJ motor endplate dispersion ratio, consistent with previous findings. USV training did not affect fiber size within the TA muscle.

Conclusions: This study demonstrated that 8 weeks of USV training can induce peripheral neural adaptations in the NMJ of the TA muscle in young rats. The observed adaptations suggest that vocal training is consistent with endurance-type exercise, but the adaptations occur on a longer time scale than similar adaptations in the limb muscles.

Voice therapy is a term used to describe a heterogeneous group of techniques directed at improving or eliminating the etiologic factors of the dysphonic voice by rebalancing the processes of respiration, phonation, and resonance (Stemple, Glaze, & Gerdeman, 2000). Some voice therapy techniques, such as resonant voice therapy, seek to improve vocal quality by targeting the efficiency of the voice (Titze, 2006). Other voice therapy techniques, such as the vocal function exercises, are derived from exercise physiology principles and are intended to improve laryngeal muscle strength, endurance, and coordination (Stemple, Lee, Damico, & Pickup, 1994). Although there have been many studies, both empirical and theoretical, on the effects

of voice therapy on vocal function and health of the superficial vibratory layers of the vocal folds (Speyer, 2008), little is known about how voice therapy affects the underlying neuromuscular mechanisms of the larynx.

Skeletal muscle exercise is typically categorized as either endurance training or strength training (Baechle & Earle, 2000). Endurance training involves exercises with submaximal repetitive contractions over long durations, such as long-distance running, swimming, or cycling. The resulting neuromuscular adaptations improve the muscles' abilities to extract and use oxygen, consequently increasing stamina (Baechle & Earle, 2000). In contrast, strength training consists of exercises with near maximal contractions with relatively few repetitions of short duration. The neuromuscular adaptations to strength training include preferential hypertrophy of Type II (fast-twitch) muscle fibers, consequently increasing strength (Folland & Williams, 2007). Vocal techniques such as the vocal function exercises seek to improve both laryngeal muscle strength and stamina (Stemple et al., 1994) and, therefore, do not conform to the classic limb exercise categories.

Specific muscle adaptations can be targeted during exercise by adjusting exercise dose parameters of frequency,

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Editor-in-Chief: Julie Liss

Editor: Michelle Ciucci

Received August 8, 2018

Revision received October 15, 2018

Accepted October 17, 2018

https://doi.org/10.1044/2018_JSLHR-S-18-0316

Disclosure: The authors have declared that no competing interests existed at the time of publication.

intensity, type, time, volume, and progression (American College of Sports Medicine, 2009). There has been some discussion of vibratory dose for vocal activity, but exercise dose has not been defined for vocal training programs (Roy, 2012). Therefore, it is unclear how vocal training fits into the classic definitions of “exercise,” both in terms of the type of exercise (endurance vs. strength) and the applicability of the frequency, intensity, type, time, volume, and progression parameters that define exercise dose.

Laryngeal neuromuscular adaptations to vocal exercise are unknown partly due to the difficulty in accessing and sampling the small laryngeal muscles in humans. In contrast, both functional and neuromuscular changes can be studied directly in the larger limb muscles by measuring strength and endurance as well as through muscle tissue biopsies. Fortunately, animal models allow for direct study of the intrinsic laryngeal muscles. In particular, training the ultrasonic vocalizations (USVs) of rats is emerging as a useful model for studying laryngeal neuromuscular responses to behavioral vocal training (Johnson, Ciucci, & Connor, 2013; Johnson et al., 2011).

Rat USVs as a Model for Studying Vocal Exercise

Rats produce USVs to communicate affective state in a variety of social contexts, including rough-and-tumble play, mating, fear response, or reward anticipation (Brudzynski, 2013). There are two distinct vocalization types classified by their center frequency and communicative intent: 22-kHz USVs are produced in aversive situations to indicate a negative affect, whereas 50-kHz USVs are produced in positive situations and indicate a positive affect (Brudzynski, 2013; Portfors, 2007). Rats also produce sonic vocalizations in the human range of hearing in response to nociceptive stimuli, such as fear or pain (Jourdan, Ardid, Chapuy, Eschalir, & Le Bars, 1995).

The larynx has long been established as the source of both audible and ultrasonic vocalizations in rats (Roberts, 1975a). There are several similarities in neurophysiological and anatomical features of rat and human larynges that support the use of USVs as a model for human vocalization training (Inagi, Schultz, & Ford, 1998; Riede, 2011). The rat larynx is an established model for investigating central and peripheral neuromuscular mechanisms underlying vocal behaviors (Ciucci et al., 2009; Ciucci, Vinney, Wahoske, & Connor, 2010; Inagi et al., 1998; Sanders, Weisz, Yang, Fung, & Amirali, 2001; Van Daele & Cassell, 2009). Additionally, the overall cartilaginous and muscular structures of the rat and human larynx are very similar, although there are two important differences (Inagi et al., 1998). The rat larynx contains the alar cartilage, a horseshoe-shaped cartilage located at the base of the epiglottis that forms the rostral edge of a ventral laryngeal air pouch, which is caudal to the anterior commissure of the vocal folds (Riede, Borgard, & Pasch, 2017). Additionally, the rat larynx contains two additional muscles not present in the human larynx, an alar branch of the thyroarytenoid (TA) muscle (also identified as the alar cricoarytenoid) and a superior cricoarytenoid muscle

(Inagi et al., 1998; Riede et al., 2017). The alar cartilage and the ventral pouch play an integral role in USV production.

Rat USVs are produced via a whistle mechanism within the larynx. Several different experimental approaches, including laryngeal denervation, excised larynges, and in vivo electromyography of the laryngeal muscles, have provided compelling evidence that laryngeal muscle contraction is necessary for USV production (Johnson, Ciucci, Russell, Hammer, & Connor, 2010; Riede, 2011, 2013; Roberts, 1975b). Recent work by Riede et al. (2017) suggests that vocal fold adduction creates a small hole through which an expiratory glottal air jet is formed. This air jet then passes across the opening of the ventral pouch, strikes the alar cartilage, and creates a turbulence that resonates in the ventral pouch. The frequency of the resonance depends on the size of the ventral pouch, which is likely controlled by the TA and cricothyroid (CT) muscles.

Although the precise sound source mechanism differs between rat USVs (whistle) and human phonation (vibrating tissue), rat USVs are a good model for studying the neuromuscular mechanisms underlying vocalization because both types of vocalizations are produced using laryngeal constriction, require respiratory–laryngeal coordination, and are frequency modulated through activation of the TA and CT muscles. The limitation of this model is that it cannot be used to examine changes to the superficial vibratory layers (epithelium and lamina propria). However, this limitation also is a strength, in that neuromuscular mechanisms can be explored with increased and decreased USV production without concern for how tissue vibration may affect the outer vibratory layers.

There is emerging evidence that increasing laryngeal muscle activity through behavioral vocal training or direct neuromuscular electrical stimulation can result in laryngeal neuromuscular adaptations, specifically within the TA muscle, the primary muscle of the vocal fold (Johnson et al., 2013; McMullen et al., 2011; Stemple et al., 2015). Behavioral training of rat USVs has been shown to reduce age-related USV acoustic changes and decrease neuromuscular junction (NMJ) motor endplate dispersion in the TA muscle of old, but not young, rats (Johnson et al., 2013). However, by modeling exercise with maximal chronic electrical neuromuscular stimulation of the recurrent laryngeal nerve, Stemple et al. (2015) found TA muscles increased in fiber areas in both young and old male rats. Neuromuscular stimulation produces maximal muscle contraction with a much greater intensity than is produced by voluntary muscle contraction during USV production. Therefore, intensity, a key parameter of physical exercise dose, appears to be a critical factor of vocal exercise to induce neuromuscular adaptations in the TA. Although laryngeal neuromuscular adaptations that occur in the rat model in response to increased use cannot be assumed to be identical to the adaptations in human muscles, the rat model can provide a basic understanding of laryngeal neuromuscular mechanisms that we cannot explore in human laryngeal muscles. Basic evidence from animal models can provide a foundation for understanding how vocal training (behavioral or electrically

stimulated) influences laryngeal muscle properties (Johnson, 2016). Once these basic mechanisms are elucidated in the animal model, it will guide our investigations in humans.

The goal of this study was to determine the effects of vocal exercise doses (short term/4 weeks vs. long term/8 weeks) on laryngeal function (USV acoustics) and neuromuscular mechanisms (TA muscle fiber hypertrophy and TA NMJ morphology) in young adult rats. Training targets increased for each rat by fixed percentages based on individual maximal performance during the first week of training. The TA muscle was chosen because it activates during USV production and has been studied previously, allowing comparison across studies.

We hypothesized that both short- and long-term vocal training would improve laryngeal function, as evidenced by increased acoustic intensity of USVs. Based on the hindlimb findings for NMJ morphology following exercise (Deschenes et al., 1993), we hypothesized that both 4 and 8 weeks of vocal training would result in increased motor endplate dispersion, nerve terminal area, and motor endplate area in the NMJs of the TA muscles and that only 8-week vocal training would result in muscle hypertrophy, consistent with the limb literature (Moritani & deVries, 1979).

Method

Animals

The animal use protocol was approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana–Champaign. Twenty-four 3-month-old male Long-Evans rats were obtained from Charles River Laboratories and randomly and equally ($n = 8$ per group) separated into three training groups: 4-week vocal training, 8-week vocal training, and untrained control. Based on a power analysis from our previous finding of the effect of USV training on NMJ motor endplate dispersion, a minimum of six animals per group would provide 95% power for detecting a significant change in motor endplate dispersion at an α level of .05 (Johnson et al., 2013). The number was increased to eight rats per group in this study to account for possible nonresponders to training. Only male rats were used in this study due to the hormonal influences of the estrous cycle on USV production in female rats (Thomas & Barfield, 1985).

Housing conditions and handling procedures were identical for both the vocally trained and control groups. All rats were kept on a 12/12 reversed light cycle and housed in pairs for the duration of the experiment. Rats underwent a 2-week habituation period to familiarize them to the recording chamber and female rat exposure. Rats were water restricted for 21 hr prior to training to motivate the response to the reward. During both the vocal training and control conditions, USVs were monitored visually on a real-time spectrogram using an ultrasonic recording system (Avisoft Bioacoustics).

Rats in the vocal training groups underwent vocal training 7 days per week with 1 day of rest every 14 days

to regain any lost weight secondary to possible dehydration. Using an established training paradigm, bouts of vocalizations were elicited by briefly exposing a male rat to a sexually receptive female rat in the male's home cage, allowing the male rat to mount the female, then removing the female rat from the male's home cage (Johnson et al., 2011). After removing the female, the male vocalizations were rewarded with a drink of water paired with the simultaneous click of a pen (Johnson et al., 2011). For the first week, vocally trained rats were rewarded for each bout of vocalizations for a total of 30 rewards. The total number of vocalizations produced was tallied daily for each rat. The targeted number of USVs increased each week by predetermined percentages according to each rat's averaged performance during the last 2 days of the previous week. The target number of USVs increased by 50% for Weeks 2 and 3, during which time the rats robustly responded to the training. After the first 3 weeks, the target number of USVs increased by 20% weekly due to a tapering of the training response. Each daily training session lasted no more than 10 min. If a rat did not meet the target number of vocalizations on the last 2 days of a week, the targeted number of vocalizations did not increase for the next week. Therefore, a rat's individual performance dictated its progression of targeted number of USVs.

Rats in the control group were identically exposed to the female rat but were not rewarded for vocalizing. Instead, control rats were position trained to the corner of the home cage each day. This position-training method did not progressively elicit vocalizations from the control group, as confirmed by real-time USV monitoring. Spontaneous vocalizations that occurred for control animals following female exposure progressively decreased over the course of position training. Half of the control group was position trained for 4 weeks, and the other half was trained for 8 weeks to balance the two vocal training conditions.

USV Acoustic Analysis

Baseline and posttraining USVs were recorded for 3 min using the same mating paradigm used for eliciting USVs during training (Johnson et al., 2011). USVs from baseline and posttraining data collection were analyzed using SASLab Pro (Avisoft Bioacoustics), which automatically calculated the USV parameters of duration, maximum frequency, frequency bandwidth, and maximum amplitude (Johnson et al., 2011, 2013). Using the acoustic analysis results, USVs were objectively classified as either simple or complex based on the presence or absence of frequency modulation as determined by the mean slope of each USV. USVs with a mean slope less than or equal to 0.2 kHz/ms (a relatively steady-state frequency) were classified as simple, and USVs with a slope greater than 0.2 kHz/ms were classified as complex (greater frequency modulation). This division between simple and complex vocalizations was performed because, in our previous experience, rats primarily produce complex, frequency-modulated USVs during

training (Johnson et al., 2013). Thus, our analysis focused on the complex USVs.

Muscle Analysis

Rats were euthanized via CO₂ inhalation 2 days after recording posttraining USVs. The entire larynx was excised, and the left and right vocal folds were each dissected. One vocal fold from each animal was prepared for fluorescent labeling of NMJ morphology, and the other was used for muscle fiber size immunostaining.

To prepare for NMJ morphology labeling, tissues were fixed in 4% formaldehyde for 1 hr, cryoprotected overnight in 20% sucrose solution, and then flash frozen in isopentane cooled with liquid nitrogen to just above its freezing point. Using a cryostat, tissues were sectioned at 30 μ m in the longitudinal plane along the length of the TA muscle (Johnson et al., 2013). Tissues were then rinsed in phosphate-buffered saline (PBS) twice for 5 min, permeabilized in 0.3% TritonX-100 in tris-buffered saline twice for 5 min, blocked in wash buffer (5.0% normal goat serum, 1.0% bovine serum albumin, and 0.01% Triton X-100 in tris-buffered saline) for 90 min, and incubated at 4°C overnight in two primary antibodies: α -bungarotoxin conjugated to Alexa Fluor 488 (1:1,000, Thermo Fisher Scientific) to label acetylcholine receptors and anti-synaptotagmin-2 (1:400; Zebrafish International Resources Center, University of Oregon) to label nerve terminal membranes. Tissues were then rinsed in wash buffer three times for 5 min, incubated in the secondary antibody Alexa 546 (1:500; Thermo Fisher Scientific) for 4 hr at room temperature, rinsed three times for 5 min, and cover slipped with ProLong Gold antifade reagent (Thermo Fisher Scientific).

Tissues used for muscle fiber size immunostaining were flash frozen fresh and then sectioned at 10 μ m in the coronal plane to create vocal fold cross-sections. Slides were stained for laminin, which outlines individual muscle fibers (see Figure 1). Muscle fiber immunostaining was accomplished using techniques established in hindlimb muscles (Bloemberg & Quadri, 2012). Tissues were air-dried for 10 min, blocked in 10% goat serum in PBS for 1 hr, and incubated with the primary antibody antilaminin (1:200; Sigma-Aldrich) to label muscle fiber outlines. The TA muscle has two divisions: the lateral and the medial (Rhee, Lucas, & Hoh, 2004). The lateral thyroarytenoid (LTA) muscle fibers are larger than the medial thyroarytenoid (MTA) muscle fibers, and therefore, fiber size was measured separately for each portion (Lenell & Johnson, 2017). The antibody 6H1 (1:50; Developmental Studies Hybridoma Bank) was used to label fiber Type IIx to distinguish the LTA (0% Type IIx) from the MTA (> 80% Type IIx). Tissues were then washed in PBS three times for 5 min, incubated in secondary antibodies Alexa Fluor 405 goat/antirabbit IgG (1:100; Thermo Fisher Scientific) and Alexa Fluor 594 goat/antimouse IgG1 (1:200; Thermo Fisher Scientific) for 1 hr, washed again in PBS three times for 5 min, and cover slipped with ProLong Gold antifade reagent (Thermo Fisher Scientific).

Figure 1. Muscle fibers outlined in blue in the medial thyroarytenoid and lateral thyroarytenoid of the vocal fold at 20 $\times$ magnification. Type IIx muscle fibers were stained red in the vocal fold to distinguish the medial thyroarytenoid, which is primarily composed of Type IIx muscle fibers, from the lateral thyroarytenoid, which has no Type IIx muscle fibers.

Image Analysis

A trained research assistant blind to the training group imaged 10 randomly selected NMJs from the TA muscle of each animal using a Zeiss 710 confocal microscope (Johnson et al., 2013). NMJs were randomly selected during imaging from a broad sample of tissue, not a single area, to prevent bias from a single muscle region. Three-dimensional z-stacks were collected for each animal in 0.5- μ m steps to allow for three-dimensional reconstruction and measurement of NMJ morphology. Confocal image stacks of NMJs were measured using a previously established automated custom ImageJ macro (Johnson et al., 2013). Briefly, NMJ image stacks were rotated in *x* and *y* to an approximated en face position, defined as the rotation at which the maximum area was measured on a z-projection. This position allowed for normalized measurements of presynaptic nerve terminals (area), motor endplates (area and dispersion), and presynaptic and postsynaptic overlap (see Figure 2).

Muscle fibers were imaged using a NanoZoomer Digital Pathology System (Hamamatsu Photonics) at 8 $\times$ exposure and 20 $\times$ magnification. Images were cropped and imported into a MATLAB application, a semiautomatic muscle analysis using segmentation of histology (SMASH). The SMASH application automatically segmented the laminin-stained muscle fibers from the background and removed nonfiber elements from the image (Smith & Barton, 2014). SMASH automatically measures and calculates the minimum feret diameters of each muscle fiber. Minimum feret diameter was chosen as the measure of fiber size because it is consistent regardless of muscle orientation and,

Figure 2. Representative neuromuscular junction images from control (A), 4-week (B), and 8-week (C) animals after automatic image processing and rotation to a standardized en face position. The presynaptic nerve terminal is pseudocolored green, the postsynaptic motor endplate is pseudocolored red, and the overlap between the two is indicated in yellow. The scale bar in each panel is equal to 10 μm .

therefore, accounts for the slight variations in cutting angle that are bound to occur when sectioning small muscles such as the TA (Briguet, Courdier-Fruh, Foster, Meier, & Magyar, 2004; Smith & Barton, 2014).

Statistical Analysis

All dependent variables were tested for normality using the Shapiro–Wilk normality test and for homogeneity of variances using Bartlett’s test for equal variances. Two-way repeated-measures analysis of variance (ANOVA) was used to compare the main effects of training group (4-week, 8-week, and control) and time (baseline and posttesting) and the interaction effect between these two independent variables on each USV acoustic dependent variable (maximum amplitude, maximum frequency, frequency bandwidth, and duration of USVs). To measure the differences in muscle fiber size of both the MTA and LTA muscles, the minimum feret diameters of 250 randomly sampled muscle fibers from each image were averaged to arrive at a single measurement for each rat and muscle. One-way ANOVA was used to test the effect of training group on mean fiber size and to compare the effect of training group on NMJ morphological measurements (nerve terminal area, motor endplate area, percentage of overlap, and motor endplate dispersion percentage). If statistical differences were found, post hoc testing was completed using Tukey’s honest significant difference test and effect sizes (Cohen’s *d*) with a 95% confidence interval were calculated. Linear regression was used to determine if the average maximum amplitude of the vocalizations could predict differences of neuromuscular measures.

Results

Response to Vocal Training

All trained rats progressively increased their performance each week during the vocal training, whereas the control group produced fewer vocalizations each week of training (see Figure 3). Three rats were removed from the

study, two from the 8-week group because they were aggressive and could not be housed with a cage mate and one from the 4-week group because of illness, resulting in uneven group numbers. All rats produced mostly complex USVs both at baseline and in posttesting (see Table 1). Statistical comparisons between groups were only performed on the complex vocalizations because simple USVs are rarely produced during training. Therefore, we expected any differences in USV acoustics to be evident in complex USVs.

USVs

Despite the robust training response, there were no interaction effects between training group and time point on any of the four acoustic measures, indicating there were no effects of training on USV acoustics. There was a significant main effect of training group on maximum amplitude, $F(2, 18) = 12.02$, $p < .001$. This difference appeared to be driven by greater USV amplitude in the 8-week

Figure 3. The mean and standard error of the number of vocalizations produced each week during each vocal training session (4- and 8-week groups) or the control interactions (control group). The trajectory of the weekly increase was the same for the two trained groups, although the individual variation in the number of ultrasonic vocalizations (USVs) produced increased each successive week. The control group interactions elicited few to no USVs.

Table 1. The mean percentage $\pm$ standard deviation of complex vocalizations at baseline and at posttesting.

Group	Percent complex at baseline	Percent complex at posttesting
Control	82.4 $\pm$ 11.4	91.0 $\pm$ 4.7
4-week	75.0 $\pm$ 15.7	89.6 $\pm$ 4.2
8-week	88.4 $\pm$ 8.8	92.7 $\pm$ 2.7

group both at baseline and at posttesting (see Figure 4). There were no other significant main effects of training group in any other USV measure.

There was a significant main effect of time on maximum amplitude, maximum fundamental frequency, and fundamental frequency bandwidth, but not on duration, indicating all three groups changed from baseline to posttesting. The maximum USV amplitude significantly increased

Figure 4. There were no interaction effects between training group (8-week, 4-week, and control) and time point (baseline or postexperiment) in any of the four ultrasonic vocalization acoustic measures, indicating no effect of training on ultrasonic vocalization acoustics. Data are shown as mean and standard error. Amplitude was measured in decibels (full scale), meaning all recorded amplitudes were negative relative to the maximum amplitude of the recording system, which was defined as 0 dB.

from baseline to posttesting by a mean of 2.5 dB for all groups, $F(1, 18) = 17.92, p < .001$. The maximum fundamental frequency and fundamental frequency bandwidth significantly decreased by 3.3 and 2.6 kHz, respectively, $F(1, 18) = 15.75, p < .001$; $F(1, 18) = 4.54, p = .047$. USV duration did not change significantly from baseline to posttesting, $F(1, 18) = 3.16, p = .09$.

Muscle Fiber Size

Due to problems with tissue processing, group sizes were reduced in this measure (control: $n = 5$, 4-week: $n = 6$, 8-week: $n = 6$). Despite the smaller-than-planned samples, all data passed the tests for normality and homogeneous variance. Overall, the LTA muscle fiber size was larger than the MTA muscle fibers across all training groups (see Figure 5). One-way ANOVA revealed no significant differences in muscle fiber size between the control and training groups in the LTA, $F(2, 14) = 0.05, p = .95$, or the MTA, $F(2, 14) = 1.57, p = .24$, indicating there was no effect of vocal training on muscle fiber size.

NMJs

The only measure of NMJ morphology that differed significantly between groups was motor endplate dispersion (see Table 2). Post hoc testing revealed that endplate dispersion of the 8-week training group was less than the control group, $p = .02$. There were no differences between the 4-week and control groups $p = .53$, or the 4- and 8-week groups, $p = .16$. These results were supported by effect size calculations that showed a large effect between the control and 8-week groups with a confidence interval not crossing 0, $d = 1.61 [0.12, 3.10]$. Effect size confidence intervals crossed 0 in the comparisons between the control and 4-week groups, $d = 0.53 [-0.20, 1.75]$, as well as between the 4- and 8-week groups, $d = 1.22 [-0.25, 2.69]$.

Linear regression showed a significant inverse relationship between the average maximum USV amplitude and NMJ motor endplate dispersion ratio (see Figure 6).

Figure 5. There were no differences in muscle fiber size between training groups within the lateral thyroarytenoid (LTA) muscle or the medial thyroarytenoid (MTA) muscle. Data are shown as mean and standard error.

Table 2. The mean $\pm$ standard deviation of neuromuscular junction (NMJ) morphology parameters and analysis of variance results between the three training groups.

NMJ parameters	Control ($n = 8$)	4-week ($n = 7$)	8-week ($n = 6$)	<i>F</i>	<i>p</i>
Nerve terminal area (μm^2)	153 $\pm$ 29.7	176 $\pm$ 33.1	142 $\pm$ 36.2	1.91	.18
Motor endplate area (μm^2)	148.5 $\pm$ 57.0	178 $\pm$ 41.4	143 $\pm$ 43.8	1.03	.38
Overlap (%)	83 $\pm$ 7.1	82 $\pm$ 3.0	84 $\pm$ 4.1	0.15	.86
Motor endplate dispersion (%)	36 $\pm$ 9.1	32 $\pm$ 7.6	23 $\pm$ 6.0	4.66	.02

Note. Between-groups and within-group degrees of freedom are 2 and 18, respectively, for all tests.

This relationship indicates rats that produced vocalizations with a greater amplitude had a smaller motor endplate dispersion within the TA muscle.

Discussion

The major finding of this study was that long-term (8-week) vocal training resulted in peripheral neural adaptations as evidenced by decreased motor endplate dispersion. This NMJ adaptation was not observed in the short-term (4-week) training group. In our previous study of USV training, we also found decreased motor endplate dispersion in the TA muscles of trained old rats, but not in trained young rats (Johnson et al., 2013). A key difference in the current study is the individualized training goal based on each rat's performance. In the previous study, all rats had the same daily vocalization target, which may have limited performance of high vocalizers or may have been an unattainable goal for low vocalizers. Based on our current results, individualizing training (increasing goals based on performance) appears to be an important consideration for inducing neuromuscular response to training. Furthermore, our results suggest that a longer training plan (8 weeks) is

required to evoke a neuromuscular response in the laryngeal muscles relative to the limb muscles. In the hindlimb muscles, exercise-induced changes at the NMJ have been reported after only 1 month of exercise (Andonian & Fahim, 1988). This longer training plan requirement may be because our vocal training protocol was limited to a period of 10 min per day or because the contractions of the intrinsic muscles during USV production are submaximal relative to activities such as swallowing (Riede, 2011).

USVs

Despite vocal training progressively increasing vocalization production, there were no statistically significant acoustic changes in USVs between groups (see Figure 3). This finding is likely related to targeting increased production of USVs rather than a specific acoustic feature. In contrast, other rat USV training studies using a parkinsonian rat model have trained increases in specific USV parameters, such as increased amplitude or complexity, resulting in changes specifically related to the targeted acoustic parameter (Ciucci et al., 2010). In our previous study of vocal training in aged rats, training rats to increase their USV production did result in an increase in USV amplitude (Johnson et al., 2013). This may have been because older rats have a smaller USV amplitude than young rats, and therefore, vocal training likely reversed this acoustic deficit.

Three of the four USV acoustic parameters changed from baseline to posttraining for all three groups with an increase in amplitude and decrease in mean frequency and frequency bandwidth. The change in amplitude may have been due to methodological reasons. The recording system was calibrated to background noise level in the room; therefore, a difference in background noise could have influenced this acoustic parameter predata to postdata collection. The recording levels were the same for all training groups at each time point and, therefore, would not have influenced group differences. Future studies will calibrate the recording system to an absolute acoustic intensity using an ultrasonic tone generator. The change in mean frequency and frequency bandwidth may reflect natural aging that occurred during the 3–4 months of habituation and training (Basken, Connor, & Ciucci, 2012). Interestingly, duration appeared to decrease in both the control and 4-week groups but increase in the 8-week group. These changes, however, were small and not statistically significant.

Figure 6. Plot of the regression analysis between maximum ultrasonic vocalization amplitude at postexperiment recordings and neuromuscular junction motor endplate dispersion ratio in the thyroarytenoid muscle. The model demonstrated a significant negative relationship, $F(1, 18) = 6.52$, $p = .02$, with an R^2 of 26%. Each point represents data from one animal (x = untrained control group, open circle = 4-week trained group, filled circle = 8-week trained group). USV = ultrasonic vocalization.

Muscle Fiber Size

TA muscle fiber hypertrophy was not observed in either vocal training group. This finding contrasts with the hypertrophy observed after simulated vocal exercise using neuromuscular electrical stimulation of the TA muscle (Stemple et al., 2015). These different findings are not altogether surprising, given that the reported neuromuscular electrical stimulation protocol maximally excited the laryngeal muscles, resulting in a higher intensity of contraction than is possible with behavioral training.

One caveat to our findings was the small sample size due to tissue processing errors, which may have underpowered our analysis of fiber size differences between groups. Another limitation of the fiber size analysis was the lack of consideration of individual fiber types when analyzing fiber size. Only fiber staining for Type IIx was conducted to differentiate the LTA from the MTA. Other fiber types were not stained because fiber type transformations were not hypothesized to occur as a result of vocal training. However, future studies should consider labeling all five types of muscle fibers to fully evaluate more the effects of training on fiber size in the laryngeal muscles.

NMJ

Changes in the size of the NMJ, including motor endplate dispersion, occur throughout the life span in response to increased and decreased muscle activity (Wilson & Deschenes, 2005). NMJ morphology also differs between muscle fiber type, although we did not consider this factor in the current study because the rat TA is entirely composed of fast-twitch muscle fibers (Rhee et al., 2004). High-intensity endurance exercise in the limb muscles causes expansion of the postsynaptic compartment and increased motor endplate dispersion (Neto, Ciená, Anaruma, Souza, & Gama, 2015). In general, NMJs increase in size in response to both endurance and strength training (Andonian & Fahim, 1988; Deschenes et al., 1993, 2000). However, high-intensity endurance training results in greater synaptic dispersion, whereas low-intensity training results in compact, symmetrical synapses (Deschenes et al., 1993). Although vocal training in targets progressed 20%–50% each week, the reduced motor endplate dispersion is indicative of an NMJ morphological change more consistent with low-intensity endurance training (Deschenes et al., 1993).

The changes in NMJ morphology in the current study suggest that our behavioral vocal training model may be more consistent with low-intensity endurance training rather than strength training. However, the absence of change in muscle fiber size and the reduction in NMJ motor endplate dispersion do not definitively characterize vocal training as an endurance activity. Much more work is needed to understand how vocal activity fits into the continuum of strength and endurance training. Future investigations should investigate endurance-type adaptations, such as increased mitochondrial size and density, increased capillarization in the muscle, and increased oxidative enzyme concentrations

(Baechle & Earle, 2000). Additionally, because other intrinsic laryngeal muscles such as the CT and the alar portion of the TA are implicated as critical to modulating the frequency of USVs, future research should also investigate neuromuscular adaptations of other intrinsic laryngeal muscles in response to vocal training (Riede, 2011; Riede et al., 2017). Our method of dissecting the whole TA allowed for easy identification of the MTA and LTA in the coronal sections used for muscle fiber analysis but made it difficult to distinguish the MTA from the LTA in the transverse orientation used for NMJ imaging. Although NMJs were randomly sampled from a large area to guard against bias from a single region, it may be that differences in fiber size between the LTA and MTA affected our NMJ morphology findings. In future studies, we recommend collecting sections of a hemilarynx (not excising the vocal fold itself) to maintain cartilaginous landmarks to better identify the LTA and MTA in the transverse plane.

In both the current study and our previous study of vocal training in old rats, the average maximum amplitude of USVs was inversely related to motor endplate dispersion. One caveat is that rats in the 8-week training group had a statistically greater USV amplitude at baseline relative to the other training groups. It was not possible, however, to assess whether the 8-week group also had less motor endplate dispersion prior to vocal training. Nevertheless, this repeated finding suggests there is a relationship between NMJ morphology and USV production. Continued research is necessary to further elucidate the relationship between the TA muscle and USV amplitude.

Conclusions

This study demonstrated that 8 weeks of vocal training can change NMJ morphology in young rats. Differences in NMJs were not noted in the 4-week animals, and therefore, the neuromuscular effects of vocal training may be delayed relative to the limb muscle's response to exercise. Furthermore, these neuromuscular adaptations suggest vocal training is a low-intensity, endurance-type exercise rather than a resistance-type exercise, although much more research is needed to verify and expand on our nascent results. This study further demonstrated that both time and progression are important parameters for influencing muscle response to vocal training and that long-term vocal training may positively influence the neural underpinnings of vocal production in an animal model. These results have begun to provide a basic understanding of laryngeal neuromuscular response to vocal exercise in ways that are currently impossible to study in humans.

Acknowledgments

This study was supported by Grant K23DC014517 from the National Institute on Deafness and Other Communication Disorders (awarded to A. M. Johnson, PI) and a New Investigators Research Grant from the American Speech-Language-Hearing Foundation (awarded to A. M. Johnson, PI). This work was completed

at the University of Illinois at Urbana–Champaign. The authors would like to thank members of the University of Illinois at Urbana–Champaign Voice Laboratory for contributing to data collection and analysis: Alexander Contreras, Jenessa Seymour, Jacklyn Nation, and Anna Fischer. The images were a collection at the Core Facilities at the Carl R. Woese Institute for Genomic Biology.

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