Effects of Age and Exercise on Neuromuscular Junction Plasticity  
in Muscles of Swallowing and Voice

by

Aaron Matthew Johnson

A dissertation submitted in partial fulfillment of  
the requirements for the degree of

Doctor of Philosophy  
(Communicative Disorders)

at the

UNIVERSITY OF WISCONSIN-MADISON  
2012

Date of final oral examination: 07/19/12

The dissertation is approved by the following members of the Final Oral Committee:  
Nadine P. Connor, Associate Professor, Communication Sciences and Disorders  
Michelle R. Ciucci, Assistant Professor, Communication Sciences and Disorders  
Susan Thibeault, Associate Professor, Division of Otolaryngology-Head and Neck Surgery  
Gary G. Weismer, Professor, Communication Sciences and Disorders  
Nathan V. Welham, Assistant Professor, Division of Otolaryngology-Head and Neck Surgery
Abstract

Sarcopenia, the loss of muscle mass and strength with aging, affects skeletal muscle throughout the body, including muscles in the tongue and larynx. These changes negatively impact muscle function and have been implicated as major contributors to swallowing and voice disorders in elderly people. One mechanism of sarcopenia may be age-related changes in neuromuscular junction (NMJ) morphology and physiology. Progressive resistance exercise training in the limb musculature has been found to prevent and/or reverse the effects of sarcopenia and reduce age-related changes at the NMJ. However, the impact of exercise on the aging muscles of the cranial sensorimotor system is unknown.

This research investigated the effects of age and exercise on NMJ morphology in one muscle of swallowing (genioglossus/GG) and one of voice (thyroarytenoid/TA) using two different models of exercise in a rat: bilateral neuromuscular electrical stimulation (NMES) and behavioral vocalization training. Additionally, the behavioral impact of the vocal training was assessed through acoustic analysis of ultrasonic vocalizations (USVs).

Results showed both models of exercise reduced age-related changes in NMJ morphology. In the GG, the relationship between motor endplates and nerve terminals was negatively affected by age but was restored with NMES. The mean and variability of motor endplate volume in old rats also decreased with NMES. In the TA, aging was
associated with increased pre-synaptic remodeling and motor endplate instability; vocal training reduced motor endplate instability and decreased variability in NMJ morphology measures. Acoustic analysis showed USV amplitude was smaller with age, but this was mitigated with vocal training. Significant correlations were found between USV acoustics and NMJ morphology in the TA, indicating changes in vocal function are related to changes in underlying neuromuscular mechanisms.

This dissertation demonstrated how two models of exercise reduced the effects of age on NMJs in muscles of swallowing and voice. Additionally, it was the first study to show vocal training impacts both vocalizations and laryngeal neuromuscular adaptations. This research has provided meaningful findings that can be used to develop hypotheses to test in human participants and pave the way for evidence-based interventions.
Acknowledgements

The completion of this Ph.D. dissertation and degree marks the end of a long journey and the beginning of a whole new set of adventures. I would not have arrived at this pivotal moment without significant support from many wonderful colleagues, friends, and family members. I am deeply indebted and grateful to them all. In particular, I would like to thank the following:

My advisor, Dr. Nadine Connor, for taking a chance on a singer who had never stepped into a lab, knowing when I needed to be challenged and when I needed support, and being not only a mentor, but also a friend.

My dissertation committee, Drs. Michelle Ciucci, Susan Thibeault, Nathan Welham, and Gary Weismer, for their collegial support and generous insight and guidance not only with this research but with my training as a clinician and academician.

My colleagues in the doctoral program and in the Connor and Ciucci labs; in particular, Kelsey Beach for her rat handling assistance, and Allison Schaser for her hours at the bench and in the scope.

Lance Rodenkirch and the W.M. Keck Laboratory for Biological Imaging for helping me see things I had never before imaged (or imagined).
Glen Levenson for completing the statistical analyses on study A and for helping me tell my story while sticking to the facts in study B.

The National Institute on Deafness and Other Communication Disorders of the National Institutes of Health for their support by grants R01DC008149 & R01DC005935 (awarded to Dr. Nadine Connor), and grant T32DC009401 (awarded to Dr. Diane Bless).

All my parents, natural, step- and in-law, for supporting me and my family, both emotionally and financially, and for always lifting me up so I can reach for my dreams.

Most of all, my wife, Cheryl, and our children, Noah, Gideon, and Eve, for keeping me grounded and balanced, giving me space and time to work, and reminding me to play.
# Table of Contents

Abstract i  
Acknowledgements iii  
Table of Contents v  
List of Tables viii  
List of Figures ix  

## Chapter 1: Introduction; Background and Significance

1.1 Statement of the problem 1  
1.2 Aging, swallowing, and voice 3  
   1.2.1 Normal aging swallow 3  
   1.2.2 Normal aging voice 4  
   1.2.3 Age-related disorders 10  
   1.2.4 Clinical treatments for dysphagia and dysphonia 13  
1.3 Aging and the neuromuscular system 16  
   1.3.1 Muscle atrophy and sarcopenia 16  
   1.3.2 NMJ anatomy and physiology 17  
   1.3.3 Agrin 19  
   1.3.4 Aging results in denervation-like changes at the NMJ 21  
   1.3.5 Exercise and aging 22  
1.4 The rat as a model for cranial muscle exercise 24  
   1.4.1 Exercise models 24  
   1.4.2 The rat tongue 25  
   1.4.3 The rat larynx 27  
   1.4.4 Ultrasonic vocalizations 28  
1.5 Hypothesis 33  
1.6 Study Overviews 34
Chapter 2: Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue (study A)

2.1 Introduction 37
2.2 Methods 40
   2.2.1 Animal Subjects and Experimental Design Overview 41
   2.2.2 Electrode Implantation and Stimulation Protocol 41
   2.2.3 Immunohistochemistry 42
   2.2.4 Confocal Microscopy 43
   2.2.5 Image Processing 43
   2.2.6 Statistical Analysis 45
2.3 Results 46
   2.3.1 Qualitative Morphologic Measurements 46
   2.3.2 Quantitative Morphologic Measurements 46
   2.3.3 Relationship between Nerve Terminal and Motor Endplate Volumes 48
   2.3.4 Comparison of Two- and Three-Dimensional Measures 53
2.4 Discussion 54
   2.4.1 Effects of Aging 54
   2.4.2 Effects of NMES 55
   2.4.3 Clinical Implications 57
   2.4.4 Relationship between Two- and Three-Dimensional Measures 58

Chapter 3: Effects of vocal training on rat ultrasonic vocalizations and neuromuscular junction morphology in the aging rat larynx (study B)

3.1 Introduction 63
   3.1.1 Background and significance 63
   3.1.2 Hypothesis 65
   3.1.3 Specific aims 65
3.2 Methodology 67
   3.2.1 Experimental overview 67
   3.2.2 Behavioral training 68
   3.2.3 USV analysis 72
   3.2.4 Tissue collection and immunohistochemistry 74
Chapter 4: Overall Conclusion

4.1 Comparison of aging and exercise in tongue and larynx 128
4.2 Physiological and functional consequences of alterations in NMJ morphology 135
4.2 Conclusion 137

References 138
List of Tables

Table 1. Main effects of age and training on USVs 95
Table 2. Differences in NMJs between lateral and medial TA muscles 102
Table 3. Main effects of age and training on NMJs in the lateral TA 103
Table 4. Main effects of age and training on NMJs in the medial TA 104
List of Figures

**Figure 1.1.** Box and whisker plots of the ultrasonic output from the adduction conditions. The box contains the interquartile range (IQR) of the data with the median data point indicated by a black dot. The whiskers extend to the last observation within 1.5 times the IQR. An asterisk indicates a significant difference from the open glottis condition (p=0.05). Reprinted with permission from Johnson, et al. (2010).

**Figure 2.1.** Box-and-whisker plots of (A) endplate volumes, (B) nerve terminal volumes, and (C) concentration ratios by age and stimulation group. The box contains the interquartile range (IQR) of the data, and the whiskers extend to the last observation within 1.5x the IQR. The open circles are observations beyond 1.5x the IQR. Note the log-transformed y-axes of the volume plots.

**Figure 2.2.** Two-dimensional projections of neuromuscular junctions from control rats in the (A) young adult and (B) middle-aged groups, demonstrating the decrease in the middle-aged motor endplate (red) size relative to the young adult motor endplates. NMJs from: (C) old control and (D) stimulation rats demonstrating the decrease in motor endplate size associated with stimulation treatment in the old group (scale bar = 10 µm).

**Figure 2.3.** Plots of the regression lines of the relationship between nerve terminal and endplate volume for each animal. Each box presents data from one rat. Dots within each box represent individual NMJs. Units for all boxes are the same and are shown on the bottom left panel. Note the log scale on both axes.

**Figure 2.4.** Means and standard errors of the slopes of the regression lines between log-transformed nerve terminal and endplate volumes in each age and treatment group. In old stimulation rats, a positive mean slope was significantly different from that observed for the old control rats (p=0.03).

**Figure 2.5.** Box-and-whisker plots of the $R^2$ values from the regressions between two- and three-dimensional measures.
Figure 2.6. Two-dimensional projections of a three-dimensional image stack of a motor endplate, demonstrating the orientation dependence of two-dimensional measurements. (A) The original orientation as collected at the microscope, and (B) after rotating the image stack 90° on the y-axis. The stained area in (A) is 43% smaller than the stained area in (B) (scale bar = 10 µm).

Figure 3.1. Spectrogram of representative USVs from the 4 classifications: (A) & (B) simple (short & long durations), (C) & (D) frequency modulated (FM) (small & large bandwidths), (E) harmonic, and (F) step. For acoustic analysis, FM, harmonic, and step USVs analyzed together as “complex”.

Figure 3.2. Photomicrographs of maximum z-projections from unmixed spectral confocal microscope image stacks demonstrating the 4 structures in the NMJ. (lateral TA from a rat in the old trained group) (scale bar = 5 µm).

Figure 3.3. Maximum z-projections of confocal image stacks showing examples of (A) an NMJ with no qualitative signs of pre-synaptic remodeling from an old trained rat, (B) axon withdrawal from 2 motor endplates from a young trained rat (note axon bundle on top right but no axon extending to either motor endplate), (C) a Schwann cell projection (arrowhead) from an NMJ from an old trained rat, and (D) an axon sprout (arrowhead) from an NMJ from an old control rat (scale bars = 5 µm).

Figure 3.4. Two images of the same NMJ demonstrating rotation of motor endplate (red) and nerve terminal (green) to an en face orientation to calculate synaptic overlap (yellow). (A) Original orientation collected at the microscope. The nerve terminal appears to be slightly to the left of the endplate, indicating the NMJ was imaged on its side. In this orientation, 82% of the motor endplate is overlapped by the nerve terminal. (B) View after rotating both the terminal and endplate 140° on the x-axis and 103° on the y-axis. These degrees of rotation give the maximum projected area for this particular endplate, providing an en face view. The nerve terminal now overlaps 98% of the motor endplate.
Figure 3.5. Sample image from agrin analysis. Z-projection of a 2-color confocal image stack after it was converted to binary using an intensity threshold (light blue = agrin, red = motor endplate, white = overlap). Image is from a rat in the young trained group. (scale bar = 10 µm).

Figure 3.6. Representative USVs recorded post-training demonstrating the smaller amplitude found in USVs from (A) an old control rat, compared with USVs from both (B) an old trained rat, and (C) a young control rat.

Figure 3.7. Comparison of vocalization rate pre/post intervention. Symbols above the 45° dashed line indicate an increased rate after 8 weeks relative to baseline.

Figure 3.8. Effect sizes with confidence intervals of comparisons between young and old demonstrating that the young adult control group had higher USV amplitudes than old in control group only (open circle = control group, filled circle = trained group).

Figure 3.9. Micrograph of the distribution of motor endplates in the rat TA. Widefield fluorescent image (4x objective) of a 50-µm thick transverse section from a rat larynx. Acetylcholine receptor clusters in the motor endplates are labeled with Alexa Fluor 488 conjugated α-bungarotoxin. Note the difference in the distribution between the horizontal endplate band in the lateral TA compared with the diffuse distribution along the length of the medial TA (scale bar = 1mm).

Figure 3.10. Micrographs of motor endplates (red) and nerve terminals (yellow) from rats in (A) the young trained group and (B) the old control group, demonstrating the larger volume in both the motor endplate and nerve terminal in the old group compared with the young group, as well as the increased en face dispersion and fragmentation in the old control group (scale bars = 5 µm).

Figure 3.11: Effect sizes with confidence intervals of comparisons between young adult and old within each experimental group (open circle = control group, filled circle = trained group), demonstrating axon withdrawal was less in the young adult control group than in the old control group, but greater in the young adult trained group than in the old trained group. This observation was not statistically significant in group comparisons.
Figure 3.12. Maximum z-projection images demonstrating the difference in en face dispersion between motor endplates from (A) an old rat in the trained group and (B) an old rat in the control group. In image B, note the greater unstained black space between the red-stained acetylcholine receptor clusters compared with image A. Also note the increased fragmentation of the motor endplate from the old control rat (scale bars = 5µm).

Figure 3.13. Significant interaction of age and training on en face dispersion in the lateral TA; within the control group, the old group had a higher en face dispersion than the young adult group. There was no difference between age groups within the trained group. Data are shown as mean and standard error.

Figure 3.14. Plot of residuals showing training significantly decreases variability in the old group (range of residuals in old trained is less than old control) in all plotted variables except fragmentation in the medial TA, which shows the opposite effect. (L-TA = lateral TA; M-TA = medial TA).

Figure 3.15. USV peak amplitude was inversely related to NMJ en face dispersion. Amplitude is reported in dBFS, with a range from -96 minimum to 0 dB maximum.

Figure 3.16. USV peak amplitude was inversely related to percentage of fragmented endplates in NMJs. Amplitude is reported in dBFS, with a range from -96 minimum to 0 dB maximum.

Figure 3.17. USV duration was positively related to the percentage of fragmented endplates in NMJs.

Figure 3.18. Comparison of the average number of vocalizations per hour during a 5-hour recording session showing the increased number of vocalizations in doubly-housed rats when recorded together, compared with the same rats (rat A and rat B) when recorded separately. The singly-housed rat (recorded alone) had the lowest number of vocalizations.

Figure 4.1. Comparison of motor endplate volume between muscles. Note the y-axis is log-transformed to facilitate easier visual comparison of outliers.

Figure 4.2. Comparison of nerve terminal volume between muscles. Note the y-axis is log-transformed to facilitate easier visual comparison of outliers.
1.1 Statement of the problem

Aging is associated with declines in neuromuscular function, including reduced muscle strength, decreased muscle contraction speed, and increased muscle fatigue (Doherty, 2003). This steady decline begins in earnest in the sixth decade, when isometric muscle strength begins to decrease approximately 1.0–1.5% per year, and is linked to a decrease in muscle mass (Vandervoort, 2002). This loss of muscle mass with aging and the cellular and molecular processes underlying this loss are collectively known as "sarcopenia" (Lang et al., 2010; Rosenberg, 1997). Sarcopenia has been reported as a possible etiology for age-related deficits in swallowing and voice (Lundy, Silva, Casiano, Lu, & Xue, 1998; McMullen & Andrade, 2006; Ota, Connor, & Konopacki, 2005; Schwarz, Thompson, Connor, & Behan, 2009).

Swallowing and voice disorders (dysphagia and dysphonia) in elderly people negatively impact quality of life, present both physical and mental health risks, and are common; it was reported that up to one-third of elderly were experiencing a problem with either swallowing or voice when interviewed (Roy, Stemple, Merrill, & Thomas, 2007a; Roy, Stemple, Merrill, & Thomas, 2007b). Sarcopenia in the tongue muscles likely underlies swallowing problems seen in elderly people, such as decreased oropharyngeal pressures and temporal deficits (Clark, Henson, Barber, Stierwalt, & Sherrill, 2003; Humbert et al., 2009; Mortimore, Fiddes, Stephens, & Douglas, 1999; Ney, Weiss, Kind,
Sarcopenia in the laryngeal muscles is associated with age-related dysphonia and may also contribute to dysphagia by compromising laryngeal closure through glottal incompetence (Hendricker, deSilva, & Forrest, 2010; Malmgren, Fisher, Bookman, & Uno, 1999; Rodeno, Sanchez-Fernandez, & Rivera-Pomar, 1993; T. Sato & Tauchi, 1982; T. Suzuki et al., 2002; Tateya et al., 2010; Tiago, Pontes, & do Brasil, 2007). Therefore, understanding the mechanisms of sarcopenia in the muscles of swallowing and voice has important clinical implications.

Behavioral training and exercise programs to treat age-related dysphonia and dysphagia have been developed, although the effects of these treatments on the cranial neuromuscular system are not fully understood (Ney et al., 2009; J. Robbins et al., 2005; Sauder, Roy, Tanner, Houtz, & Smith, 2010; Spielman, Ramig, Mahler, Halpern, & Gavin, 2007; Stemple, Lee, D'Amico, & Pickup, 1994; Stemple, Glaze, & Klaben, 2009; Yeates, Molfenter, & Steele, 2008). One reason for this is the challenge in obtaining human samples for histopathological and biochemical examination; it is possible to biopsy the limb musculature without disrupting function, but the small size and inaccessibility of the muscles of swallowing and voice limit these types of invasive procedures. Animal models can solve this problem. Therefore, it is critical to find appropriate animal models of cranial muscle exercise to explore underlying mechanisms of sarcopenia and the effects of intervention on the senescent voice and swallow.
1.2 Aging, swallowing, and voice

1.2.1 Normal aging swallow

**Swallowing function**

Age-related changes within the oropharyngeal swallow have been found in otherwise healthy elderly people (Ekberg & Feinberg, 1991). These changes include decreases in oral strength and pressure, a delay and overall increase in the time of the oropharyngeal swallow, and increases in cortical activation during swallowing relative to normal young adults. (Humbert et al., 2009; Ney et al., 2009; Nicosia et al., 2000; Teismann et al., 2010). The overall slowing of the oropharyngeal swallow may put elderly people at risk for aspiration. Therefore, advanced age may increase the risk for dysphagia, even in asymptomatic elderly people (Ekberg & Feinberg, 1991; J. Robbins, Levine, Wood, Roecker, & Luschei, 1995).

**Age-related tongue changes**

Functional changes with advanced age have been described in the human tongue, including decreased maximum tongue strength and increased tongue fatigability (Mortimore et al., 1999; Youmans, Youmans, & Stierwalt, 2009). Studies of oral pressure during isometric tongue press showed decreased isometric tongue pressures in elderly males despite adequate pressures during swallowing (Nicosia et al., 2000; J. Robbins et al., 1995). Examination of elderly human cadaveric tongues has shown morphological changes, such as a decrease in the thickness of tongue epithelium, atrophy of the salivary glands, and a decrease in muscle fiber diameter (Nakayama, 1991), as well as
replacement of muscle with fatty tissue (Bassler, 1987). Decreases in both objective and subjective measures of tongue strength are related to deficits in the oral phase of swallowing (Clark et al., 2003). Therefore, it is likely that age-related changes in the muscles of the tongue contribute to changes in swallowing function in elderly people.

1.2.2 Normal aging voice

Auditory-perceptual

The perception of a speaker's voice is a good indicator of the speaker's chronological age. Based on recordings of sustained vowels and/or speech samples (both reading and spontaneous speech), listeners are reliable and confident when classifying a speaker as young or old, determining the decade of a speaker's age, or directly estimating the speaker's age (Jacques & Rastatter, 1990; Neiman & Applegate, 1990; Ryan & Burk, 1974; Shipp & Hollien, 1969). Age estimates made from speech samples are as accurate as those made from a photograph (Krauss, Freyberg, & Morsella, 2002). The primary perceptual vocal features associated with an "old" voice are alterations in pitch and vocal quality; specifically, vocal tremor, tension, strain, and/or breathiness indicate increased chronological age (Harnsberger, Brown, Shrivastav, & Rothman, 2010; Hartman, 1979; Ryan & Burk, 1974). In addition to vocal features, cues in the speech signal are related to the perception of advancing chronological age, such as a slow rate of articulation and imprecise consonants (Hartman, 1979; Ryan & Burk, 1974). Although the severity of these perceptual features do not seem to progress linearly with chronological age, it is clear that old and young voices are perceived as different (Eppley & Mueller, 2001).
There are several factors that affect the accuracy of age estimations by listeners. Listeners more accurately judge the age of speakers who are of a similar age to themselves; younger listeners tend to underestimate the age of older speakers and older listeners have a more difficult time discriminating the age of younger speakers (Huntley, Hollien, & Shipp, 1987). Further study, however, has shown that listeners of all ages underestimate the age of older speakers (Eppley & Mueller, 2001). The disparity in these findings may result from 2 factors related to the sex of the listener and speaker: 1) listeners tend to be more accurate when listening to speakers of the same sex, and 2) a perceptual aftereffect has been shown in that listeners perceive voices as older after listening to a young voice; this aftereffect is stronger when listeners and speakers were of the same sex (Zaske & Schweinberger, 2011). Additionally, a history of voice training and professional voice use, as well as general physical fitness and activity level lowers the perceived age of a speaker (Prakup, 2012; A. Xue & Muelle, 1997). Overall, the evidence supports that changes in perceptual voice features are indicative of a speaker's age, and that these perceptual changes are linked to changes in the acoustic signal (Gorham-Rowan & Laures-Gore, 2006; Ryan & Burk, 1974).

**Acoustics and aerodynamics**

Extensive study has been done on the acoustic changes in the aging adult voice (for review, see Baken (2005) and Linville (1996)). In general, acoustic measures of the normal aging adult voice (adults without complaints of dysphonia) are considered to be "worse" than those of the younger voice (S. Xue & Deliyski, 2001). With age comes
alterations in speaking fundamental frequency (W. S. Brown, Morris, Hollien, & Howell, 1991; Harnsberger, Brown, Shrivastav, & Rothman, 2009; Hollien & Shipp, 1972), reduction in intensity and range (Teles-Magalhaes, Pegoraro-Krook, & Pegoraro, 2000), decrease in the harmonics to noise ratio (Ferrand, 2002; Gorham-Rowan & Laures-Gore, 2006), increases in perturbation measures (Gorham-Rowan & Laures-Gore, 2006; Linville & Fisher, 1985; Wilcox & Horii, 1980), and alterations of vocal tract resonances (Linville & Rens, 2001; Linville, 2002). In a clinical composite acoustic measure, the Dysphonia Severity Index, normative values are negatively affected by age (Hakkesteegt, Brocaar, Wieringa, & Feenstra, 2006).

A consistent finding in acoustic studies of the aging voice is increased variability in measurements both within and between subjects with age (Gorham-Rowan & Laures-Gore, 2006; Morris & Brown, 1994; Stathopoulos, Huber, & Sussman, 2011). One interpretation of this finding is that aging negatively impacts the precision of control over the vocal mechanism (Baken, 2005). This increased variability may in and of itself be a hallmark of the aging voice and makes it difficult to define the effects of "normal" aging. This variability may also be a reflection of how acoustic measures, like perceptual measures, may be more of a reflection of physiologic age versus chronological age, since they are affected by overall health and well-being and history of professional voice use (W. S. Brown, Morris, Hicks, & Howell, 1993; Chodzko-Zajko & Ringel, 1987; Orlikoff, 1990; Ramig & Ringel, 1983).
It is likely that the effects of age on acoustic measures are linked to changes in laryngeal aerodynamics (Awan, 2006; Higgins & Saxman, 1991). However, the effect of age on laryngeal aerodynamics is not well understood, perhaps because of the reported variability in aerodynamic measurements; high intersubject variability both within and between age groups is a consistent finding in studies of aging and laryngeal aerodynamics (Goozee, Murdoch, Theodoros, & Thompson, 1998; Sapienza & Dutka, 1996). These changes in vocal function as measured by acoustics and aerodynamics are assumed to be due to age-related changes in laryngeal biology and physiology (Gorham-Rowan & Laures-Gore, 2006; Hollien & Shipp, 1972).

**Visual-perceptual**

Laryngoscopic examination of the larynx also reveals age-related changes. In a study comparing acoustic measures with laryngoscopic findings between young and old adults, older men were found to have a higher fundamental frequency and increased vocal fold atrophy compared with younger men; older women were found to have a lower fundamental and vocal fold edema (Honjo & Isshiki, 1980). A common appearance of the older larynx on stroboscopic examination is an anterior or spindle-shaped gap during phonation (Linville, 1992; Pontes, Yamasaki, & Behlau, 2006). This is often attributed to bowing of the vocal folds and has been confirmed by examination of extirpated cadaveric larynges (Mueller, Sweeney, & Baribeau, 1985). The degree of bowing, however, is not necessarily directly related to the size of the glottal gap (Bloch & Behrman, 2001). Other age-related changes in videostroboscopic measures include increased aperiodicity of
vibration, slower speed of vocal fold opening during phonation, alterations in the mucosal wave, and protuberance of the vocal prominences (Biever & Bless, 1989; Murty, Carding, & Kelly, 1991; Pontes, Brasoлотto, & Behlau, 2005).

**Biology and physiology**

Many biological and physiological changes have been reported in the senescent larynx. The age-related changes in perceptual, acoustic, aerodynamic, and visual-perceptual measures outlined above are likely due to alterations in the underlying biology and physiology of the voice.

The lamina propria determines the biomechanical properties of the vocal folds which, in turn, determine the vibratory characteristics. Age-related changes in the lamina propria have implications for altering the biomechanics of the vocal fold. Rheological study of the human vocal fold mucosa has shown an increase in stiffness and viscosity of the vocal fold with age (Chan & Titze, 1999). Histological studies of older larynges have shown alteration of the layered structure of the lamina propria, with differences between the sexes (Hammond, Gray, & Butler, 2000; Hirano, Kurita, & Sakaguchi, 1989; Madruga de Melo et al., 2003; Ximenes Filho, Tsuji, do Nascimento, & Sennes, 2003). While these studies are in agreement that the lamina propria is altered with aging, the way in which it is altered is not agreed upon; for example, some report thickening and edema of the superficial layer (M. Hirano et al., 1989), while others have found thinning of the superficial layer with aging (Ximenes Filho et al., 2003). Individual variability may
account for these discrepancies, as well as variations in measurement techniques. Despite the varied conclusions of the studies, there is agreement that these alterations of the layered structure with age are related to alterations in the amount, distribution, and structure of elastin, collagen, and hyaluronic acid in the lamina propria, and that the functional consequence of this is an alteration in the biomechanical properties of the vocal fold (Ding & Gray, 2001; Gray, Titze, Alipour, & Hammond, 2000; S. Hirano, Bless, del Rio, Connor, & Ford, 2004; K. Sato & Hirano, 1997; K. Sato, Hirano, & Nakashima, 2002). It is likely that the acoustic changes observed in older voices, particularly the changes in fundamental frequency, have much to do with the alterations in stiffness of the vocal fold as opposed to a change in vocal fold mass (Titze, 2011).

Vocal fold fibroblasts are responsible for managing the proteins in the vocal fold lamina propria (Gray, 2000). A reduction of fibroblast activation with aging has been suggested, but this reduction may be reversed through treatment with basic fibroblast growth factor (S. Hirano et al., 2004; K. Sato & Hirano, 1995). Gene expression analysis from human vocal fold fibroblasts has shown an aging effect in the mRNA expression levels of collagen and elastin, with a decrease in levels of collagen mRNA and an increase in levels of elastin mRNA (X. Chen & Thibeault, 2008). Examination of fibroblasts in vitro may provide good indicators of physiologic age, such as differences in fibroblast morphology, proliferation rate, and telomere length (X. Chen & Thibeault, 2008; Thibeault, Glade, & Li, 2006).
Reduced activation of the thyroarytenoid (TA) muscle as measured by electromyography has been implicated as a likely contributor to the typical acoustic and perceptual changes in the aging voice (Baker, Ramig, Luschei, & Smith, 1998). The TA and its innervation have been the focus of age-related neuromuscular changes in the larynx (for review, see Thomas (2008)). As with other skeletal muscles, the TA muscle atrophies with age, likely due to a loss of muscle fibers and a conversion of muscle fiber type from fast to slow fibers (Malmgren et al., 1999; Rodeno et al., 1993; T. Sato & Tauchi, 1982; T. Suzuki et al., 2002; Tiago et al., 2007). Aging results in denervation-like changes in the laryngeal neuromuscular system, including reduction in myelinated nerve fibers, ultraterminal sprouting of axons at neuromuscular junctions, and remodeling of motor endplate morphology (Connor, Suzuki, Lee, Sewall, & Heisey, 2002; McMullen & Andrade, 2009; Mortelliti, Malmgren, & Gacek, 1990; Perie, St Guily, Callard, & Sebille, 1997). The functional consequences of these neuromuscular changes are decreases in muscle strength, contraction speed, and fatigue resistance.

1.2.3 Age-related disorders

Dysphagia

The prevalence of dysphagia in elderly people has been estimated to be between 14 – 33% (Kawashima, Motohashi, & Fujishima, 2004; Roy, Stemple, Merrill, & Thomas, 2007a). The true prevalence of dysphagia, however, is likely unknown, as symptoms are underreported due to a misconception among elderly people that dysphagia is a normal and, therefore, acceptable sequela of aging (Bloem et al., 1990; P. H. Chen,
Dysphagia presents significant, even mortal, health risks, including risks of aspiration pneumonia, malnutrition and weight loss, and depression (Cabre et al., 2010; Ekberg, Hamdy, Woisard, Wuttge-Hannig, & Ortega, 2002; Roy, Stemple, Merrill, & Thomas, 2007a). Therefore, studying age-related changes in the neuromuscular system that contribute to functional changes in the swallow is critical to help understand how to design therapeutic interventions to restore swallowing function.

**Presbyphonia**

The age-related voice changes outlined above are reported from studies of normal elderly people and do not constitute a voice disorder (dysphonia) in and of themselves. When changes in vocal quality and/or vocal ability interfere with communication, they become dysphonia. The prevalence of chronic dysphonia in elderly people of any etiology is reported to be between 20-28%. (Golub, Chen, Otto, Hapner, & Johns, 2006; Roy, Stemple, Merrill, & Thomas, 2007b; Turley & Cohen, 2009). Dysphonia due to age-related vocal changes is commonly referred to as "presbyphonia".

Presbyphonia is defined as a combination of videostroboscopic and auditory-perceptual findings: vocal fold bowing and a breathy, weak vocal quality (Kendall, 2007; Woo, Casper, Colton, & Brewer, 1992). The incidence of presbyphonia as the primary cause of dysphonia in patients over 60 years of age has been estimated to be between 4 - 30% (Hagen, Lyons, & Nuss, 1996; Kandogan, Olgun, & Gultekin, 2003; Woo et al.,
Presbyphonia is a “diagnosis of exclusion” (Woo et al., 1992); that is, other vocal pathologies unrelated to age-related changes must first be ruled out. Commonly diagnosed causes of voice disorders in patients over 60 that are not necessarily related to aging include benign or malignant vocal fold lesions, central neurological disorders, functional dysphonia, laryngopharyngeal reflux, muscle tension dysphonia, and paresis (Gregory, Chandran, Lurie, & Sataloff, 2011; Hagen et al., 1996; Kandogan et al., 2003; Woo et al., 1992). It also may be hard to distinguish between age-related changes and the effects of vocal disuse, as elderly patients presenting to the voice clinic with signs of possible age-related changes, such as muscle deconditioning and vocal fold bowing, rate themselves as low talkers and having quiet voices (Bastian & Thomas, 2000).

Dysphonia in elderly people has a significant negative impact on quality of life (Costa & Matias, 2005; Golub et al., 2006; Roy, Stemple, Merrill, & Thomas, 2007b; Turley & Cohen, 2009; Verdonck-de Leeuw & Mahieu, 2004). Elderly people with dysphonia have increased communication difficulties because of deficits in vocal quality and vocal effort, report lower (worse) scores on the voice-related quality-of-life (V-RQOL) measure, and tend to avoid social gatherings (Golub et al., 2006; Verdonck-de Leeuw & Mahieu, 2004). Social withdrawal is not surprising, given that listeners rate speakers with older-sounding voices as having negative personality traits (Mulac & Giles, 1996). As with voice disorders in the general population, both health- and voice-related quality of life measurements are not directly related to the severity of vocal deficits as measured by acoustic analysis; the impact of a voice disorder on quality of life may be
greater or less than the abnormalities in the acoustic signal (Plank, Schneider, Eysholdt, Schutzenberger, & Rosanowski, 2011; Schneider, Plank, Eysholdt, Schutzenberger, & Rosanowski, 2011). Therefore, defining dysphonia in the elderly population should include quality of life measures and not just measures of vocal function.

1.2.4 Clinical treatments for dysphagia and dysphonia

Behavioral clinical interventions for age-related dysphagia and dysphonia use task-specific exercises to strengthen the muscles involved in swallowing and voice, improve coordination and timing, and/or provide compensatory strategies. In dysphagia intervention, surface neuromuscular electrical stimulation (NMES) of the neck musculature is sometimes used as a treatment for dysphagia, with or without behavioral muscle strengthening exercises (Carnaby-Mann & Crary, 2007; Carnaby-Mann & Crary, 2008; Clark, Lazarus, Arvedson, Schooling, & Frymark, 2009; Freed, Freed, Chatburn, & Christian, 2001; Shaw et al., 2007).

Surface NMES is common in physical therapy as an adjunct to volitional exercise and is often useful when patients are unable to achieve adequate levels of muscle contraction voluntarily (Paillard, 2008). Surface NMES has been used in a similar fashion in swallowing rehabilitation with some success, although results from clinical studies have been mixed and the technique has become controversial (Carnaby-Mann & Crary, 2007; Carter, 2011; Clark et al., 2009; Humbert, 2011). Further research on the clinical
utility of NMES in dysphagia rehabilitation and its effect on neuromuscular mechanisms in the tongue is essential to determine if the technique is a safe and effective intervention.

**Exercises to strengthen the tongue**

Age-related changes in tongue strength, as described above, likely contribute to dysphagia in the elderly population (Clark et al., 2003). Principles of strength-training exercise from the more robust physical therapy literature may be adapted to dysphagia therapy in elderly people to strengthen the muscles involved in swallowing, including the tongue (Burkhead, Sapienza, & Rosenbek, 2007; J. Robbins et al., 2008). Exercise focused on increasing tongue strength is viable in the elderly population (Ney et al., 2009; J. Robbins et al., 2005). Initial studies have shown normal elderly research participants increase tongue strength using a resistance exercise paradigm (J. Robbins et al., 2005). Additionally, elderly people with dysphagia can use tongue exercise to not only increase tongue strength, but also improve on functional measures of swallowing (Yeates et al., 2008).

**Use of voice therapy to treat presbyphonia**

There is clinical support for the use of voice therapy and vocal exercise programs to treat age-related dysphonia (Sauder et al., 2010; Spielman et al., 2007; Stemple et al., 1994; Stemple et al., 2009). However, there is little evidence of how, if at all, vocal exercise affects the laryngeal neuromuscular system (Thomas et al., 2008). Understanding the mechanisms of exercise on the muscles of the upper airway is critical
to developing treatments to prevent and/or reverse the effects of age-related voice disorders. Much of what we know about voice disorders in the elderly population and the effects of therapy comes from retrospective chart reviews (Berg, Hapner, Klein, & Johns, 2008; Gartner-Schmidt & Rosen, 2011). Controlled, prospective studies are needed to better understand the effects of age and behavioral intervention on the voice. Studying the laryngeal muscles in humans is difficult and often impractical due to their small size and inaccessibility. Therefore, a behavioral animal model would be beneficial. This research includes an innovative approach that explored how vocal exercise affected the neuromuscular system of the aging larynx at the connection between nerves and muscles (Study B).

*Professional voice and vocal aging*

The role of vocal exercise and training as a means to improve vocal quality and ability is supported by improved perceptual and acoustic measures of professional voice users (Prakup, 2012; W. S. Brown et al., 1993). Singers are not immune from the effects of age; perceptual features specific to the singing voice are affected by aging, such as a slowing of the vibrato rate (Sundberg, Thörnvik, & Söderström, 1998). However, when compared with age-matched non-singers, older adult singers exhibit improved acoustic performance (less jitter and increased intensity), experience less change in speaking fundamental frequency, are perceived as younger, and maintain vocal stability longer (Prakup, 2012; Boone, 1997; W. S. Brown, Morris, & Michel, 1990; W. S. Brown et al., 1991; W. S. Brown et al., 1993). These findings from professional singers suggest vocal
training and/or singing training may be a way to preserve vocal function in the aging adult voice.

1.3 Aging and the neuromuscular system

1.3.1 Muscle atrophy and sarcopenia

To fully understand how sarcopenia impacts the cranial muscles involved in swallowing and voice, it is important to understand the mechanisms underlying age-related muscle changes. There are several hypothesized cellular and molecular causes of sarcopenia, including an imbalance in protein synthesis and degradation, a decrease in insulin growth factor, an increase in inflammatory factors, oxidative mitochondrial damage, and a decrease in the number and function of muscle satellite cells (for review, see Lang (2010)). Another primary cause of sarcopenia is denervation and loss of primary motor neurons, resulting in a decline in the number of motor units, conversion of fast to slow muscle fibers, and a clustering of muscle fiber types within the muscle (Carlson, 1995; Lang et al., 2010; Larsson, 1995; Vandervoort, 2002).

Due to the varied factors contributing to the development of sarcopenia, it can be difficult to define what exactly is meant by this term (Waters, Baumgartner, Garry, & Vellas, 2010). It has been suggested that sarcopenia may be considered a 'geriatric syndrome' as opposed to an age-related disease; that is, sarcopenia is not purely a result of the aging process but of a combination of etiologic factors (Cruz-Jentoft, Landi, Topinkova, & Michel, 2010). For example, variability and task-dependence of fatigability
suggests central mechanisms, such as central drive and changes in metabolic capacities, contribute to muscle fatigue (Allman & Rice, 2002).

### 1.3.2 NMJ anatomy and physiology

The neuromuscular junction is the interface between the peripheral motor neuron and the muscle. It traditionally is thought to consist of three primary cellular components: terminal Schwann cells, nerve terminals, and motor endplates (Sanes & Lichtman, 1999). Recently, a fourth component, dubbed the kranocyte, has been proposed as a capping cell that possibly assists with synaptic regeneration (Court et al., 2008).

On the pre-synaptic side are the terminal Schwann cells and motor neuron terminals. The terminal Schwann cells are mature, non-myelinating, perisynaptic glial cells that guide synapse formation and reinnervation and modulate neurotransmitter release (Sugiura & Lin, 2011). The main purpose of the motor neuron terminal is to store and release the neurotransmitter acetylcholine (ACh) that is required for synaptic activation and consequent muscle contraction. The synaptic vesicles within the nerve terminal contain ACh in discrete packets called quanta that dock with the nerve terminal membrane to release their contents into the synaptic cleft upon signaling from the motor neuron (Calakos & Scheller, 1996). The number of quanta released is called the quantum release and under normal circumstances is much greater than is required for muscle activation (Engel, 2008). The difference between what is required for muscle activation
and the excess quanta released is referred to as the safety factor of neuromuscular transmission (Engel, 2008; Wood & Slater, 2001).

When the ACh enters the synaptic cleft, it travels a short distance to the motor endplate that lies opposite the nerve terminal along the deep invaginations of the postsynaptic membrane. At the top of the junctional folds of the membrane is a high concentration of acetylcholine receptors (AChRs) (between 15,000 - 25,000 per μm²) (Salpeter & Loring, 1985). Five transmembrane polypeptides make up the AChR, two of which bind with ACh to open the receptor and allow an influx of Na+ into the endplate, depolarizing the membrane and signaling the muscle to contract (Alberts et al., 2002).

The adult NMJ is a dynamic structure that is constantly undergoing small-scale remodeling but maintains its overall structure throughout its development and maturity (Balice-Gordon & Lichtman, 1990; Sanes & Lichtman, 1999). The adult motor endplate appears pretzel-like in its morphology and is closely aligned with the presynaptic nerve terminal (Balice-Gordon & Lichtman, 1993; Slater, 1982). Successful neurotransmission depends on this precise spatial arrangement of the pre- and postsynaptic components (Alberts et al., 2002). Change in the degree of synaptic overlap has implications for neurotransmission success; less overlap would imply a longer path for ACh diffusion and possibly increase the chance of failure (Prakash, Miller, Huang, & Sieck, 1996). Additionally, the size and complexity of the NMJ depend on the muscle fiber type on which it lies; NMJs on slower type I and IIa fibers are smaller, less complex, and have
greater synaptic overlap than NMJs on type IIX and IIB fibers (Deschenes, Covault, Kraemer, & Maresh, 1994; Ogata & Yamasaki, 1985; Prakash et al., 1996). There may be a functional explanation for this. Smaller NMJs are found on smaller-sized type I and IIA muscle fibers that have a lower threshold of activation and, thus, are innervated by motor neurons with a smaller discharge potential. (Deschenes et al., 1994; Prakash et al., 1996)

Age-related changes in NMJ morphology have been shown to precede atrophic changes of muscle fibers (Deschenes, Roby, Eason, & Harris, 2010). Therefore, this research is focused on the impact of exercise on age-related changes at the NMJ.

1.3.3 Agrin

The synaptic basal lamina contains many elements that play crucial roles in development, maintenance, and reinnervation of the neuromuscular junction (Alberts et al., 2002; Sanes, 2003). Many secreted factors have been identified as critical to the differentiation of the pre- and post-synaptic compartments of the NMJ; the primary factor implicated in the aggregation of AChRs into the motor endplate is agrin (Johnson-Venkatesh & Umemori, 2010). Agrin is a heparin sulfate proteoglycan transported by the motor neuron to the basal lamina of the muscle (Daggett et al., 1996; Gesemann, Denzer, & Ruegg, 1995; Groffen et al., 1998; McMahan, 1990). Through interaction with its primary receptor, muscle-specific kinase (MuSK), agrin signals AChR aggregation during development (Bezakova, Rabben, Sefland, Fumagalli, & Lomo, 2001; Ngo, Noakes, & Phillips, 2007; Pun et al., 2002). Additionally, the presence of agrin in the
A mature motor endplate is critical for maintaining AChR clusters, as agrin has been reported to counteract the dispersal effect the neurotransmitter ACh exerts as it is released from the AChR (Misgeld, Kummer, Lichtman, & Sanes, 2005).

Agrin also is involved in the denervation/reinnervation process. Agrin protects denervated NMJs by maintaining endplate structure in the absence of synaptic activity, increasing the chance of successful reinnervation (Pun et al., 2002). Denervated muscles show an increase in agrin sensitivity outside of existing NMJs, paving the way for the formation of new AChR clusters (Bezakova & Lomo, 2001; Cohen, Rimer, Lomo, & McMahan, 1997). During reinnervation of NMJs after nerve damage, projections of perisynaptic Schwann cells express agrin to promote the formation of new AChR clusters (Z. Feng & Ko, 2008). Therefore, agrin is an important factor to consider when studying changes in motor endplate morphology.

Although agrin has been well-studied in the developing, adult, and denervated NMJ (Kummer, Misgeld, & Sanes, 2006; Sanes, 2003), the role of agrin in the aging NMJ is unknown. Recently it has been suggested that induced agrin loss through proteolytic cleavage results in muscle fiber and NMJ changes that are phenotypically similar to sarcopenia, implying agrin loss is one of the precursors to sarcopenia (Butikofer, Zurlinden, Bolliger, Kunz, & Sonderegger, 2011). Additionally, some of the changes in the senescent NMJ, such as extrajunctional receptors and endplate fragmentation, indicate the aggregating function of agrin decreases with age (Sanes &
Lichtman, 1999). Therefore, it is reasonable to hypothesize that age-related changes in agrin likely contribute to observed age-related changes in NMJ morphology.

**1.3.4 Aging results in denervation-like changes at the NMJ**

Although the NMJ remains remarkably stable throughout maturity, many age-related changes in NMJ morphology have been found with senescence. These changes occur suddenly and mimic changes observed when either the muscle and/or peripheral nerve are damaged, as in denervation (Li, Lee, & Thompson, 2011; Rich & Lichtman, 1989; Rosenheimer, 1990). These senescent changes precede clinical signs of sarcopenia and may be caused by oxidative stress, mitochondrial dysfunction, or loss of primary motor neurons, resulting in larger motor units and either permanent or temporary denervation of NMJs (Deschenes et al., 2010; Jang & Van Remmen, 2011).

Senescent NMJs have similar morphological features as denervated young adult NMJs, such as extra-junctional processes of terminal Schwann cells that are extended to guide attempts at reinnervation by neuronal sprouts. (O'Malley, Waran, & Balice-Gordon, 1999; Son & Thompson, 1995a; Son & Thompson, 1995b). Other signs of this denervation-reinnervation process in aging muscles, including the laryngeal muscles, are increased innervation ratios, increased size of motor units, and transformation of muscle fiber types (Larsson & Ansved, 1995; Malmgren et al., 1999; McMullen & Andrade, 2009; Rodeno et al., 1993; Takeda, Thomas, & Ludlow, 2000). The senescent NMJ exhibits other morphological changes, including fragmentation of the motor endplate,
loss of AChRs, reduced numbers of nerve terminals, and loss of alignment between pre- and postsynaptic components (Balice-Gordon, 1997a; Connor et al., 2002; Elkerdany & Fahim, 1993; Fahim & Robbins, 1982).

The mature NMJ is a reliable synapse that is relatively unaffected by small-scale morphological remodeling, but the more significant age-related morphologic changes seen with senescence have physiological significance that are linked to deficits in neuromuscular function (Alshuaib & Fahim, 1990; Deschenes, 2011; McMullen & Andrade, 2009; Slater, 2008a). The age-related morphological changes outlined above likely disrupt the relationship between the pre- and post-synaptic components of the NMJ, leading to increased synaptic failure and inefficiency (Balice-Gordon, 1997a; N. Robbins, 1992; Smith, 1979; Smith, 1984). Examples of this are age-related decreases in quantal release (the amount of neurotransmitter released with each nerve impulse) and in the safety factor (the amount of neurotransmitter release above and beyond what is needed to activate muscle contraction) (Wood & Slater, 2001).

1.3.5 Exercise and aging

Research has shown that the decrease in strength in aging muscles may be reversed and/or prevented by exercise (Fiatarone & Evans, 1993). In aging muscles, exercise increases muscle mass and strength, and increases capacity for anabolic processes through upregulation of protein synthesis, a sign of muscle growth and maintenance (Vandervoort, 2002). Physical activity positively modulates mechanisms of
anabolism, energetic metabolism, anti-oxidant response, and musculotendinous architecture, and negatively modulates mechanisms of inflammation, catabolism, and denervation (Pillard et al., 2011). Exercise and activity cannot stop the biological aging process, but they can minimize the physiological effects (Chodzko-Zajko et al., 2009).

In the limb musculature, exercise can reduce, prevent, and/or reverse age-related changes of NMJ morphology and physiology (Alshuaib & Fahim, 1990; Andonian & Fahim, 1987; Fahim, 1997). Alteration of muscle use patterns has an effect on NMJ morphology and, consequently, neuromuscular transmission; increased muscle use (exercise) enhances neuromuscular transmission, while decreased muscle use has the opposite effect (Deschenes et al., 1994; Wilson & Deschenes, 2005). These morphological effects on the NMJ occur both pre- and post-synaptically and manifest differently in young, middle-aged, and old age groups as well as in different muscles and muscle fiber types (Andonian & Fahim, 1987; Deschenes & Wilson, 2003; Deschenes, Roby, & Glass, 2011; Sanes & Lichtman, 1999). In young animals, exercise appears to increase synaptic remodeling, whereas in old animals exercise appears to reduce the signs of denervation, such as nerve terminal sprouting, that appear with old age (Andonian & Fahim, 1987; Deschenes et al., 1994; Fahim, 1997; Wilson & Deschenes, 2005).
1.4 The rat as a model for cranial muscle exercise

1.4.1 Exercise models

Models of progressive resistance exercise can be classified into 4 categories; resistance training, electrical stimulation, compensatory overload, and chronic stretch (Lowe & Alway, 2002). Another form of exercise involves low intensity but high repetition of a movement. This research examines two models of exercise in the cranial muscles; electrical stimulation of the tongue muscles using NMES of the hypoglossal nerves, and low intensity high repetition exercise of the laryngeal muscles through voluntary behavioral vocal training. Typical resistance training employs high-intensity, low-repetition exercise (Vincent et al., 2002). However, low-intensity training with high repetition has been shown to be as effective as high-intensity resistance training in increasing muscle strength and hypertrophy in elderly women and men (Taaffe, Pruitt, Pyka, Guido, & Marcus, 1996; Vincent et al., 2002).

The behavioral vocal training model used in this research can be classified as a low-intensity, high repetition resistance exercise. The resistance component is not applied externally, as in weight training, but may be a derivative of vocalization biomechanics. That is, during vocalization (both human and rat), TA contraction is opposed by contraction of the cricothyroid muscle and the cartilaginous attachments of the TA, thus creating an isometric contraction which increases the stiffness of the muscle and provides internal resistance (Titze, 2000).
The advantage of the vocalization training model used in this research is that it is analogous to typical low-intensity limb muscle training programs in elderly humans (Vincent et al., 2002), improving the model's external validity. The primary disadvantage in the context of the current research is that rats must be motivated through deprivation and/or reward and these motivators may influence the effects of training by inducing stress (Lowe & Alway, 2002). NMES does not rely on motivation or cooperation of animals and allows for precise control over the quantity and repeatability of muscle recruitment. The disadvantages of NMES are that it often requires repeated anesthesia, the difference in muscle recruitment pattern from voluntary muscle contraction, and the influence of the simulation parameters on the outcomes (Lowe & Alway, 2002).

1.4.2 The rat tongue

The rat tongue is an established model for studying neuromuscular changes resulting from age and exercise (Connor, Ota, Nagai, Russell, & Leversen, 2008; Hodges, Anderson, & Connor, 2004; Nagai, Russell, Jackson, & Connor, 2008; Ota et al., 2005; Schwarz et al., 2009). It is important to study the tongue directly and not make inferences from the limb musculature, because the tongue displays different age-related changes than hindlimb muscles (Connor et al., 2008). The aging rat tongue demonstrates decreased tetanic tension and increased contraction and recovery times while maintaining adequate maximal strength and fatigue resistance (Nagai et al., 2008; Ota et al., 2005). NMJs in the aging rat tongue demonstrate signs of denervation-like remodeling (Hodges et al., 2004). These changes at the NMJ of the genioglossus muscle may be related to a
decrease in the number of primary dendrites of hypoglossal motoneurons (Schwarz et al., 2009). These findings indicate changes in the peripheral neuromuscular system may contribute to age-related deficits in tongue strength and function and, consequently, swallowing function.

Two models of exercise, behavioral training and neuromuscular electrical stimulation (NMES), have been used to explore the effects of age and exercise on muscle function and physiology in the rat tongue. Behavioral training through a licking/tongue press paradigm increased maximum tongue force as well as the size of muscle fiber cross-sectional area at all ages (young adult, middle-age, and old) (Connor et al., 2009). Direct stimulation of peripheral nerves through electrode implantation (NMES) has been used to model exercise in the limb musculature (Widick, Tanabe, Fortune, & Zealear, 1994). This NMES model was recently used to study sarcopenia in the tongue musculature through stimulation of the hypoglossal nerve; the first results from this research showed that NMES is associated with a reduction in some age-related changes in tongue muscle contractile properties, muscle fiber type, and neuromuscular junction morphology (study A) (Connor et al., in press; Johnson & Connor, 2011).

The rat tongue has also been used to investigate the effects of age on changes in muscle fiber type distribution, neurotrophic factors, and orolinguinal function, (Schaser, Wang, Volz, & Connor, 2011; Schaser, Stang, Connor, & Behan, 2012; Volz et al., 2007; Zhang, Bethel, Smittkamp, & Stanford, 2008), as well as deficits in the tongue
neuromuscular system related to Parkinson disease (Ciucci & Connor, 2009; Ciucci et al., 2011). Most recently, functional videofluoroscopic measures of swallowing have shown the effects of age and Parkinson disease on swallowing manifest similarly in the rat as in humans (Russell, Ciucci, Hammer, & Connor, in press). Therefore, the rat is an established model for studying changes in the tongue related to swallowing and disease.

1.4.3 The rat larynx

The rat larynx is an established model for studying laryngeal neuromuscular biology (Inagi, Schultz, & Ford, 1998). Structurally, the rat larynx is very similar to the human larynx in its cartilaginous and muscular framework (Smith, 1977), although some differences exist, such as proportional size of the arytenoid cartilages. Also different from the human larynx is the presence of an additional anterior cartilage superior to the vocal folds, the laryngeal alar cartilage, as well as two additional muscles, the alar cricoarytenoid muscle, thought to perform a similar function as the aryepiglottis muscle, and a superior cricoarytenoid muscle, thought to function similarly to the interarytenoid muscle (Inagi et al., 1998).

One of the early motivations for using the rat larynx was to understand the effects of botulinum toxin (BT) injections into the laryngeal musculature for the treatment of voice disorders such as spasmodic dysphonia (Inagi et al., 1998; Inagi, Connor, Ford et al., 1998; Inagi, Connor, Schultz et al., 1998; Inagi et al., 1999). One of these studies established the location of motor endplates within the larynx and defined two major
compartments of the thyroarytenoid in the rat; the medial and lateral portions corresponding with the vocalis and muscularis portions of the TA in humans (Inagi et al., 1998).

In the aging rat larynx, senescence is associated with decreased laryngeal kinematics during resting breathing, reduced capillary surface area and branch points in the TA, and a shift to slower contracting muscle fiber types in the TA (Russell, Nagai, & Connor, 2008; T. Suzuki et al., 2002; T. Suzuki, Connor, Lee, LeVerson, & Ford, 2002). In both the human and rat larynx, aging impacts laryngeal NMJ morphology in a manner similar to denervation; however, laryngeal NMJs have features different than NMJs in the limb musculature and, therefore, require direct study (Connor et al., 2002; Z. Feng, Zhang, Ralston, & Ludlow, 2012; Gambino, Malmgren, & Gacek, 1990; Malmgren et al., 1999). These results indicate aging negatively impacts the physiology and function of the rat laryngeal neuromuscular system.

Although the rat larynx is a well-established model for studying laryngeal neuromuscular biology, there have been no investigations into the effects of a behavioral intervention on peripheral laryngeal neuromuscular changes.

1.4.4 Ultrasonic vocalizations

Few animal models exist that allow for study of laryngeal neuromuscular mechanisms and vocal training (Sutton, Larson, Taylor, & Lindeman, 1973). Training
rats to increase the production and/or complexity of their ultrasonic vocalizations (USVs) provides a novel opportunity to study the effects of behavioral interventions (vocal exercise and training) on laryngeal neuromuscular mechanisms. Rats can be trained to modify their USVs through behavioral operant conditioning incorporating stimuli that elicit USVs, including mating, simulation of juvenile rough-and-tumble-play, or pharmaceutical and/or electrical brain stimulation (Burgdorf & Panksepp, 2001; Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Burgdorf et al., 2008; Ciucci, Ma, Kane, Ahrens, & Schallert, 2008; Ciucci, Vinney, Wahoske, & Connor, 2010; Johnson et al., 2011). Using these paradigms, rat USVs have been used to study a variety of correlates between the brain and behavior, including the central mechanisms underlying laughter and joy (Panksepp, 2007) and vocal deficits related to Parkinson disease (Ciucci et al., 2007; Ciucci et al., 2008).

Rat USVs are produced via a whistle mechanism created by laryngeal constriction (Johnson et al., 2010; Riede, 2011; Roberts, 1975a; Roberts, 1975b; Sanders, Weisz, Yang, Fung, & Amirali, 2001; Wetzel, Kelley, & Campbell, 1980). Acoustic studies using light gas in the vocal tract have shown the fundamental frequency of USVs increases proportionally to the light gas concentration, supporting that a whistle mechanism and not tissue vibration is the USV sound source (Riede, 2011; Roberts, 1975b). Laryngeal denervation studies show intact laryngeal innervation of both the superior and recurrent laryngeal nerve is required for normal USV production (Roberts, 1975a; Wetzel et al., 1980). Laryngeal constriction has been observed in anesthetized rats...
using laryngeal endoscopy during elicitation of USVs via periaqueductal gray area stimulation (Sanders et al., 2001). In an excised larynx model, laryngeal constriction was necessary to produce biologically relevant ultrasounds up to and above 50 kHz, the typical high frequency of range of USVs (Johnson et al., 2010). In that study, sutures were used to simulate different degrees of glottal adduction. In the suture conditions, but not in the open glottis condition, ultrasounds up to and above 50 kHz were achieved with a minimum airflow rate of at least 4 L/min and subglottal pressure of least 27 cm H2O (Figure 1.1) (Johnson et al., 2010). Therefore, there is strong support for larynx as the source of USVs.
Figure 1.1. Box and whisker plots of the ultrasonic output from the adduction conditions. The box contains the interquartile range (IQR) of the data with the median data point indicated by a black dot. The whiskers extend to the last observation within 1.5 times the IQR. An asterisk indicates a significant difference from the open glottis condition (p=0.05). Reprinted with permission from Johnson, et al. (2010).
In addition to being produced with a laryngeal constriction, USVs are also similar to human vocalizations in that they require rapid adjustments of the laryngeal muscles and control over subglottal pressure to modulate acoustic output (Riede, 2011). It follows that with these common mechanism of production, there are common peripheral and central neural pathways of control for both human and rat vocalization (Schulz, Varga, Jeffires, Ludlow, & Braun, 2005; Van Daele & Cassell, 2009). Also like human vocalizations, rat USVs exhibit age-related acoustic changes, such as decreased acoustic complexity and intensity with age (Basken, Connor, & Ciucci, 2012). Therefore, although produced via a whistle mechanism and not tissue vibrations, rat USVs are a homologous behavior to human vocalizations.

Rat USVs are typically divided into two acoustically distinct frequency categories: 22-kHz and 50-kHz (Portfors, 2007). Typical 22-kHz USVs have a flat and steady frequency and are relatively longer in duration than 50-kHz USVs (Blanchard, Agullana, McGee, Weiss, & Blanchard, 1992). The 50-kHz USVs are often more acoustically complex than the 22-kHz USVs and are usually further classified as constant frequency (flat) (Brudzynski & Pniak, 2002; Ciucci et al., 2009; Fu & Brudzynski, 1994; Wintink & Brudzynski, 2001), frequency modulated calls (FM) (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Burgdorf et al., 2007), or harmonic calls (Figure 4.1) (Ciucci et al., 2009). USVs have also been classified based on a combination of behavior and acoustic measurements, showing three distinct clusters of USVs in the 25, 40, and 60-kHz ranges that correspond to fighting, feeding, and moving, respectively (Takahashi,
An association between behavior and acoustic features of 22-kHz and 50-kHz USVs has also been well-established.

Different communicative contexts elicit either the 22-kHz or the 50-kHz USVs, implying each vocalization type has a distinct communicative purpose. USVs in the 22-kHz range are produced in aversive situations, such as in response to predation or environmental stress, and are used as an alarm call to warn conspecifics (Blanchard et al., 1992). Conversely, USVs in the 50-kHz range are associated with positive affective states, such as reward and mating (Brudzynski & Bihari, 1990; Burgdorf et al., 2008). Therefore, USVs are used for communication and can be a model to study vocalizations with communicative intent.

The ability to train rats to produce USVs, the neuromuscular mechanism of USV production in common with human vocalizations, and the communicative intent of USVs, all support the use of USVs as the basis for establishing a model of vocal exercise. That is, training rats to increase the number, loudness and complexity of USVs and, consequently, increase use of the laryngeal musculature, is a viable model of vocal exercise.

1.5 Hypothesis

The central hypothesis of this research was that increasing activation of muscles of swallowing and voice using models of exercise would reduce and/or reverse the
impact of aging at the neuromuscular junction. To test this hypothesis the effects of age and exercise on NMJ morphology in the genioglossus muscle (tongue) was examined by comparing NMJs from young adult, middle-aged, and old rats that had either received bilateral hypoglossal nerve stimulation (NMES) or had been in a control group (study A). Next, a study was performed comparing USV acoustics, NMJ morphology, and agrin localization in the thyroarytenoid muscles (larynx) in young and old rats that were vocally trained versus a control groups of young and old animals (study B).

1.6 Study Overviews

Study A: Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue

This study shows how neuromuscular electrical stimulation of the hypoglossal nerve is associated with restoration of the pre-post synaptic relationship at the NMJ in aging rats.

Abstract

Alterations in neuromuscular junction (NMJ) structure in cranial muscles may contribute to age-related deficits in critical sensorimotor actions such as swallowing. Neuromuscular electrical stimulation (NMES) is used in swallowing therapy, but it is unclear how NMJ structure is affected or if NMJ morphology is best measured in two or three dimensions. Two- and three-dimensional measurements of NMJ morphology in the genioglossus muscle were compared in rats that had undergone 8 weeks of hypoglossal
nerve stimulation vs. untreated controls. The relationship between motor endplate volume and nerve terminal volume had a mean positive slope in 90% of the young adult controls, but it was positive in only 50% of the old controls; 89% of NMES old rats had a positive slope. NMJ measurements were more accurate when measured in three dimensions. In the NMJ, aging and NMES are associated with changes in the pre- and post-synaptic relationship.

**Study B: Effects of vocal training on rat ultrasonic vocalizations and neuromuscular junction morphology in the aging rat larynx**

This study uses an animal model of vocal exercise to explore the effects of age and vocal training on rat USVs and NMJs in the thyroarytenoid muscle and their relationship.

*Abstract*

Behavioral voice therapy is a critical component in the treatment of voice disorders, but the effects of vocal exercise and training on neuromuscular mechanisms in unknown. The ultrasonic vocalizations (USVs) of rats have much in common with human vocalizations; they share central and peripheral neural pathways, are produced in the larynx by air flowing through a constricted glottis, and are modulated by rapid adjustments of the intrinsic laryngeal musculature. Therefore, training rats to increase and/or modulate their USVs can be used as a model to study the connection between vocal behavior and laryngeal neuromuscular mechanisms. Both young and old rats were
either vocally exercised or given no intervention over 8 weeks. USVs were compared between age and intervention groups both pre- and post-intervention. Changes in laryngeal neuromuscular mechanisms were assessed by measuring neuromuscular junction (NMJ) morphology in the thyroarytenoid muscle as well as the localization of agrin, a necessary component of motor endplate aggregation. Age differences were found in measurements of both USV acoustics and NMJ morphology. Vocal training reduced or eliminated some of these differences. Significant correlations between measurements of USV acoustics and NMJ morphology were found, particularly between measures of vocalization amplitude and motor endplate stability. This study is the first to examine how training rats to increase their USV production changes their vocal behavior, and how those changes correlate with underlying neuromuscular adaptations.
Chapter 2: Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue (study A)

Authors: Aaron M. Johnson and Nadine P. Connor. Published in Muscle and Nerve (Johnson & Connor, 2011).

2.1 Introduction

Dysphagia has been identified as a common and chronic problem in the aging population (Roy, Stemple, Merrill, & Thomas, 2007a). Age related changes in the tongue likely contribute to muscle weakness and fatigue, and, consequently, dysphagia (Mortimore et al., 1999; Nakayama, 1991; Nicosia et al., 2000; Palmer et al., 2008; Van Daele, McCulloch, Palmer, & Langmore, 2005). However, age-related changes in the tongue muscles and the response of these muscles to different treatments have been insufficiently studied (Connor et al., 2009; Nagai et al., 2008; Ota et al., 2005).

Age-related morphologic changes at the neuromuscular junction are similar to changes found in the denervation–reinnervation process (Balice-Gordon, 1997a; Connor et al., 2002; Larsson & Ansved, 1995). Aging changes the size, complexity, and relationship of pre- and post-synaptic neuromuscular junction (NMJ) morphology (Connor et al., 2002; Elkedany & Fahim, 1993; Hodges et al., 2004; Malmgren, Jones, & Bookman, 2001; McMullen & Andrade, 2009). The physiologic sequelae of age-related morphologic changes include reduced synaptic transmission efficiency and increased
fatigue (Balice-Gordon, 1997a; Fahim & Robbins, 1982; McMullen & Andrade, 2009; Rosenheimer & Smith, 1985; Rosenheimer, 1990). Therefore, study of NMJ morphology may provide insight into mechanisms underlying age-related muscle weakness and fatigue.

Neuromuscular electrical stimulation (NMES) is used in physical therapy and swallowing therapy to restore function and strength of impaired muscles (Braid et al., 2008; Huckabee & Doeltgen, 2007; Sheffler & Chae, 2007). In physical therapy, lower limb rehabilitation NMES in combination with voluntary exercise has been shown to be more effective than voluntary exercise alone (Paillard, 2008). A combination of voluntary exercise and NMES is also used for the treatment of dysphagia by placing surface electrodes on the neck while a patient swallows and/or performs swallow strengthening exercises (Clark, 2003). Although initial studies have shown some benefit from this approach (Carnaby-Mann & Crary, 2007) thorough evidence of its efficacy is lacking (Clark et al., 2009).

There is a paucity of research on how NMES affects underlying neuromuscular mechanisms. Although it has been shown that exercise changes the size and complexity of both pre- and post-synaptic structures of the NMJ (Andonian & Fahim, 1988; Deschenes et al., 2000; Deschenes, Tenny, & Wilson, 2006; Fahim, 1997), little is known about the effects of electrical stimulation on NMJ morphology (Eberstein & Pachter, 1986; Stanco & Werle, 1998; Tam, Archibald, Jassar, Tyreman, & Gordon, 2001).
Most of the research on the relationships among aging, exercise, and NMJ morphology has been performed in animal hindlimb muscles (Alshuaib & Fahim, 1990; Deschenes, Tenny, Eason, & Gordon, 2007; Fahim & Robbins, 1982), or the diaphragm (Prakash & Sieck, 1998), whereas little work has focused on the cranial muscles (Connor et al., 2002; Hodges et al., 2004; McMullen & Andrade, 2009). There are important structural and functional differences between limb and cranial muscles that underscore the need to directly examine muscles of the head and neck. For example, cranial muscles typically have lower innervation ratios than muscles of the hindlimb (Kanda & Hashizume, 1992; Sutlive, Shall, McClung, & Goldberg, 2000). In addition, there are different age-related changes in muscle contraction times and maximum force generation between limb and tongue muscles (Connor et al., 2008). Direct study of cranial muscles is critical to a complete understanding of age-related muscle changes in the head and neck and to interventions targeted at particular cranial functions. Inferences from hindlimb muscles, although valuable at a conceptual level, provide limited information about specific changes that likely occur in the cranial system.

Although the NMJ is a three-dimensional structure, technology has only recently allowed investigation of NMJ morphology in three dimensions (Court et al., 2008; M. Suzuki, Maruyama, Sugiura, Machida, & Miyata, 2009). Maintaining three-dimensionality may provide more precise measurements of NMJs. However, the consistency of nerve terminal and motor endplate measurements made in two dimensions
vs. three dimensions has not been examined previously, and thus conclusions regarding potential differences in measurement accuracy cannot be drawn based on available data.

The purpose of this study was to determine how chronic electrical stimulation affects two- and three-dimensional NMJ morphology in the aging rat tongue. Our hypotheses were: (1) age-related changes in NMJ morphology would be manifested as alterations in the size and relationship of motor endplates and nerve terminals; (2) these age-related changes would be reduced with chronic electrical stimulation; and (3) two- and three-dimensional measurements would correlate poorly. We tested these hypotheses by comparing two- and three-dimensional measurements of NMJs in young adult, middle-aged, and old genioglossus muscles in two groups of rats; one group had undergone 8 weeks of chronic hypoglossal nerve stimulation, and one did not receive any intervention.

2.2 Methods

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the NIH’s Guide for Care and Use of Laboratory Animals, and the animal welfare act. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison School of Medicine and Public Health.
2.2.1 Animal Subjects and Experimental Design Overview

A total of 50 male Fischer 344/Brown Norway rats are reported from three age groups: 9 months (young adult, n=18); 24 months (middle-aged, n=13); and 32 months (old, n=19). This strain of rats has a 33-month median lifespan (Turturro et al., 1999). As part of a larger study, rats within each age group were randomly assigned to a group that received 8 weeks of bilateral electrical stimulation of the hypoglossal nerves (8 young adult, 7 middle-aged, and 9 old animals) or a control group that did not receive electrical stimulation (10 young adult, 6 middle-aged, and 10 old animals).

2.2.2 Electrode Implantation and Stimulation Protocol

Animals in the stimulation group were surgically implanted with a prefabricated electrode assembly (Widick et al., 1994), consisting of a skin plug for connecting to an external stimulator, six lead wires, and two stimulation electrode cuffs placed around the hypoglossal nerve on each side. To simulate a clinical exercise paradigm used to strengthen the tongue muscles (Robbins et al., 2005) and to optimize the potential for inducing muscular adaptation, chronic stimulation was administered 5 days a week for 8 weeks. Each repetition consisted of a 1.0-s stimulation train at 40 Hz followed by 1.0 s of rest, with a 2-min rest interval between sets. Stimulation pulses of 0.2-ms duration were delivered at supramaximal intensity to recruit all muscle fibers innervated by the hypoglossal nerve (generally 300–500 lA). Supramaximal intensity was empirically determined for each animal by finding the current at which maximum tongue force was
achieved, as measured by a force transducer attached to the tongue tip, and gradually increasing the current to 1.5 times the maximum intensity.

At the conclusion of the 8-week study period, all animals were anesthetized (ketamine 70 mg/kg, xylazine 7 mg/kg) and euthanized by an overdose of Beuthanasia via intracardiac injection. The genioglossus muscle was immediately dissected, flash frozen in 2-methylbutane cooled by liquid nitrogen, and stored at 80° Celsius.

2.2.3 Immunohistochemistry

At a later time, whole muscles were fixed in 4% formaldehyde for 1 h at room temperature. Longitudinal, 50-µm-thick sections were cut from the midsection of each muscle and mounted directly onto a slide coded such that the microscopist (A.M.J.) was masked to the age and experimental group of the animals from which samples were derived.

To visualize relevant pre- and post-synaptic structures, samples were labeled with three different fluorophores according to a protocol based on prior work in this area (Balice-Gordon, 1997a; Connor et al., 2002; Hodges et al., 2004). To label acetylcholine receptors within the motor endplate, samples were incubated in 20 µg/ml of tetramethylrhodamine-conjugated α-bungarotoxin (Invitrogen/Molecular Probes, Eugene, Oregon) in phosphate-buffered saline (PBS) for 30 min at room temperature. Pre-synaptic structures were labeled by incubating the samples overnight in the following primary
antibodies diluted to final concentration in wash buffer: 1:200 anti-neurofilament (SMI 31, mouse IgG1; Covance, Emeryville, California); 1:100 anti-synaptic vesicle (SV2, mouse IgG1; Developmental Studies Hybridoma Bank, Iowa City, Iowa); and 1:250 anti–S-100, labeling both myelinating and non-myelinating terminal Schwann cells (S-100, rabbit IgG; Dako, Carpinteria, California). The next day, samples were incubated for 4 h in 1:100 Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) and 1:100 Alexa Fluor 488–conjugated donkey anti-rabbit IgG (Invitrogen/Molecular Probes).

2.2.4 Confocal Microscopy

Individual NMJs were imaged via confocal microscopy on a Radiance 2100 MP (Bio-Rad Microscience, Ltd., Hemel Hempstead, Herts, UK) using a PlanApo, 60x, oil immersion objective, with a 1.40 numerical aperture (Nikon, Melville, New York). A minimum of 10 NMJs per animal were collected. Images were collected using three different laser lines and sequential line scanning to prevent cross-talk between color channels. Image stacks with a 0.5- lm spacing and 1-lm optical section depth were collected for each of the three colors.

2.2.5 Image Processing

Image stacks were processed using ImageJ (Rasband, 1997-2009). After examining a three-dimensional reconstruction of each NMJ, an assistant (A.J.S.), blinded to age and experimental group, rated the following four qualitative measurements as
present or absent: (1) Schwann cell processes; (2) axon sprouting; (3) annular-appearing motor endplate; and/or (4) motor endplate unoccupied by a nerve terminal.

Quantitative measurements of (1) aggregate nerve terminal volume, (2) aggregate nerve terminal area, (3) motor endplate volume; (4) motor endplate area, and (5) motor endplate concentration ratio (see definition in what follows) were automatically calculated using a custom ImageJ macro (A.M.J.). First, an automatic thresholding algorithm (Auto Threshold v1.8, G. Landini, 2009) was applied to each image stack. Next, the image stacks containing the axons and synaptic vesicles and Schwann cells were combined to create an aggregate nerve terminal image stack. A convex hull algorithm (Convex Hull Plus, G. Landini, 2004) was used to calculate the total volume occupied by the stained motor endplate and its interstitial area.

Volumes of the aggregate nerve terminal, motor endplate, and convex hull image stacks were calculated by multiplying the sum of the thresholded area on each slice by the z-slice depth. Two-dimensional areas of both the aggregate nerve terminal and the motor endplate were calculated on maximum z-projections of each thresholded image stack. The maximum z-projection is a two-dimensional image with each pixel in that image representing the maximum intensity value at that pixel location over all images in the stack. The motor endplate concentration ratio was calculated by dividing the motor endplate volume by the volume of the convex hull (Deschenes et al., 2000).
A total of 529 NMJs were collected across the 50 animals studied. Results of the automated volume and area measurements were plotted for examination of outliers. When individual measurements extended greater than 2 standard deviations from the mean for any of the dependent variables, further examination of those particular images was performed; 32 images met this definition. Data from 17 images were subsequently removed from the analysis either because the image contrast was insufficient for the auto-threshold algorithm to differentiate the structure of interest from the background or because the entire NMJ was not contained within the image stack. Removal of the data was necessary, because these particular images could not be accurately measured. After removal of these outliers, a total of 512 NMJs were used in the final analysis. The median number of NMJs per animal was 10, with a range of 6–13. The median number of slices per image stack was 49, with a range of 21–89.

2.2.6 Statistical Analysis

Analysis of variance (ANOVA) was used to test age effects, stimulation treatment effects, and age x stimulation treatment interactions on volume and area of motor endplates and nerve terminals, endplate concentration ratio, and the variability (standard deviation) of the size of motor endplates and nerve terminals within each animal. Mean volume and area data were log-transformed to approximate a normal distribution. Non-transformed data were used for the comparisons of standard deviations, because log-transformation reduces heteroscedasticity. Post hoc pairwise comparisons were made between groups using the Fisher protected least significant difference tests.
All analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, North Carolina). The critical value for obtaining statistical significance was set at the $\alpha = 0.05$ level.

2.3 Results

2.3.1 Qualitative Morphologic Measurements

In the 512 NMJs evaluated, only 10 Schwann cell processes, 2 axon sprouts, and 13 annular-appearing motor endplates were observed. No motor endplates unoccupied by a nerve terminal were observed. Because these observations were so rare across age and treatment groups, further statistical analysis was not performed on these measures.

2.3.2 Quantitative Morphologic Measurements

Motor endplate volume, nerve terminal volume, and concentration ratio by age and stimulation treatment group are summarized in Figure 2.1. A significant age x stimulation treatment interaction effect was found for mean motor endplate volume ($F_{[2,44]}=4.12$, $p=0.02$), and standard deviation of motor endplate volume ($F_{[2,44]}=3.67$, $p=0.03$). Paired comparisons are described in what follows. No significant main or interaction effects were found for nerve terminal volume or endplate concentration ratio.
Figure 2.1. Box-and-whisker plots of (A) endplate volumes, (B) nerve terminal volumes, and (C) concentration ratios by age and stimulation group. The box contains the interquartile range (IQR) of the data, and the whiskers extend to the last observation within 1.5x the IQR. The open circles are observations beyond 1.5x the IQR. Note the log-transformed y-axes of the volume plots.
Fisher post hoc protected least significant difference (LSD) tests showed that mean motor endplate volume was larger in young adult control animals than in the middle-aged control animals \((p=0.04)\) (Figure 2.2), but it was not different from old control animals \((p=0.6)\). Further, old stimulation animals had a smaller mean \((p=0.02)\) and standard deviation \((p=0.01)\) for motor endplate volume than did old control animals (Figure 2.2). No significant differences were found between control and stimulated animals within the young adult and middle-aged groups. Within the stimulation treatment group, mean motor endplate volume was significantly smaller in the old stimulation animals than in the young and middle-aged stimulation animals \((p=0.05\) and \(p=0.02\), respectively).

### 2.3.3 Relationship between Nerve Terminal and Motor Endplate Volumes

The relationship between nerve terminal volume and motor endplate volume for each animal is shown in Figure 2.3. ANOVA results revealed a significant interaction effect between age and stimulation treatment for the slope of the regression lines \((F_{[2,44]}=0.66, \ p=0.03)\) (Figure 2.4). For controls, both the young and middle-aged groups had mean slopes of approximately 1.0, with positive slopes in 90\% of the young adults and in 83\% of the middle-aged animals. However, the old control animals had a mean slope of -0.43, with a positive slope in only 50\% of the animals. Fisher post hoc protected LSD tests showed the slopes of both the young adult and middle-aged control groups were significantly different than the slope of the old control group \((p<0.001\) and \(p=0.003\), respectively).
Figure 2.2. Two-dimensional projections of neuromuscular junctions from control rats in the (A) young adult and (B) middle-aged groups, demonstrating the decrease in the middle-aged motor endplate (red) size relative to the young adult motor endplates. NMJs from: (C) old control and (D) stimulation rats demonstrating the decrease in motor endplate size associated with stimulation treatment in the old group (scale bar = 10 µm).
Figure 2.3. Plots of the regression lines of the relationship between nerve terminal and endplate volume for each animal. Each box presents data from one rat. Dots within each box represent individual NMJs. Units for all boxes are the same and are shown on the bottom left panel. Note the log scale on both axes.

There was also a significant difference in mean slope between old control animals and old stimulation animals (p=0.03). The mean slope in the old stimulation animals was 0.49, and the percentage of animals with a positive slope increased to 89%. Within the young adult and middle-aged animals there was not a significant difference in slope between the stimulation treatment and control groups. The percentage of animals in the stimulation group with a positive slope was 100% in the young adult animals and 71% in the middle-aged animals. There were no significant main or interaction effects for the root-mean-square error.
Figure 2.4. Means and standard errors of the slopes of the regression lines between log-transformed nerve terminal and endplate volumes in each age and treatment group. In old stimulation rats, a positive mean slope was significantly different from that observed for the old control rats (p=0.03).
Figure 2.5. Box-and-whisker plots of the $R^2$ values from the regressions between two- and three-dimensional measures.
2.3.4 Comparison of Two- and Three-Dimensional Measures

Results of linear regressions of motor endplate volume and area across animals were variable (mean $R^2=0.58$, SD=0.22), with a wide range of $R^2$ values (0.03–0.95). A similarly variable relationship was found between nerve terminal volume and area (mean $R^2=0.62$, SD=0.23, range 0.001–0.98). Data from these regressions are summarized in Figure 2.5.

ANOVA results for area measurements were different than those for volume measurements reported previously in this study. In contrast to the significant differences found using volume measurements, differences in mean motor endplate area between the young adult and middle-aged control groups were not statistically significant ($p=0.08$), nor was the standard deviation of the motor endplate area between old control and old stimulation animals ($p=0.8$). Conversely, a significant main effect for stimulation treatment was found for terminal area ($p=0.04$), but not for terminal volume ($p=0.14$).

Two- and three-dimensional measurements of endplate concentration ratio were moderately correlated (mean $R^2=0.32$) with high variability (SD=0.26) and a wide range of $R^2$ values (<0.01–0.95). Neither analysis showed statistically significant main or interaction effects for aging or stimulation.
2.4 Discussion

This study has shown that NMJ morphology is altered in an extrinsic muscle of the aging rat tongue and that chronic electrical stimulation is associated with a reduction in age-related changes. In addition, the correlation between two- and three-dimensional measurements of NMJ morphology is unreliable and, therefore, can lead to different conclusions about the effects of aging and electrical stimulation on NMJ structure.

2.4.1 Effects of Aging

The age-related changes in NMJ morphology found in this study have been demonstrated in two ways: changes in the size of motor endplates, and a breakdown of the relationship between motor endplate and nerve terminal size. We found smaller motor endplates in middle-aged animals than in young adult animals, but no difference between young adult and old endplate volumes. This non-linear trend is consistent with a previous study from our laboratory in which a nonsignificant decrease in receptor area was found between young adult (9 months) and old (36 months) animals (middle-aged animals were not included in that study) (Hodges et al., 2004). Rosenheimer & Smith (1985), found a similar non-linear trend in age-related effects with a peak in age-related NMJ changes at middle age (28 months) and a subsequent reduction in old animals (31 months).

Nonlinear age-related changes in muscle physiology have also been reported, with an increase in time to peak contraction and half-relaxation time in the extensor digitorum longus and plantaris muscles of 28-month-old, but not 36-month-old rats, when compared with young rats (M. Brown & Hasser, 1996). Therefore, the effects of aging on skeletal
muscles, including NMJ morphology, do not follow a linear progression, and studies of multiple age groups are needed to fully understand how aging affects the senescent NMJ.

The relationship between motor endplate and nerve terminal size was disrupted in old animals. In the young and middle-aged control animals, the mean positive slope of the regression line of approximately 1.0 between motor endplate volume and nerve terminal volume indicated that, as motor endplate volume increased, nerve terminal volume also increased proportionally. In contrast, the negative mean slope of the regression line found in the old control animals indicates that the relationship between motor endplate volume and nerve terminal volume was not maintained with increasing age. Because this inverse relationship was only found in the old, untreated control animals, a deviation in the size relationship between pre- and post-synaptic sides of the NMJ may be a factor that underlies the age-related decline in neuromuscular function in the genioglossus muscle of the tongue.

2.4.2 Effects of NMES

Chronic electrical stimulation appeared to restore the relationship between pre- and post-synaptic morphology in old animals to a state similar to that found in young adult and middle-aged animals. In contrast to the negative regression slope in the old control animals, old animals that received NMES had a mean positive regression slope that was similar to the slopes found in young adult and middle-aged control animals. This may have resulted from the reduced mean and standard deviation of motor endplate
volume in the old animals with NMES. Interestingly, NMES did not significantly affect the size of motor endplates or the relationship between motor endplate and receptor volumes in young adult or middle-aged animals. Therefore, as with age-related changes in NMJ morphology, the effects of NMES varied within different age groups.

Variations in NMJ morphology affect the reliability and efficiency of synaptic function (for review, see Slater (2008)). Increased post-synaptic folding has been shown to amplify the effect of acetylcholine on muscle activation by doubling the safety factor for neuromuscular transmission (Wood & Slater, 1997). In addition, it has been suggested that the degree of postsynaptic folding is inversely related to the size of the motor endplate (Slater, Lyons, Walls, Fawcett, & Young, 1992; Slater, 2003). Therefore, it is possible that NMES may result in increased post-synaptic folding and improved synaptic efficiency by reducing the mean and variability of motor endplate volume in old animals. Increased synaptic efficiency could facilitate muscle contractions and reduce fatigue. There are many other factors that may contribute to the functional recovery observed with NMES. For example, NMES has been shown to alter muscle fiber phenotype expression in both cranial and limb muscles (for review, see Pae et al. (2007) and Pette and Staron (2001)), as well as increase muscle fiber cross-sectional area (Palmer et al., 2008).

It is possible that changes in the muscle fibers may have accounted for the morphologic changes observed in the NMJ. However, there is evidence that there is
independence between muscle fiber size and NMJ morphology. For example, Deschenes et al. (2000) showed that changes in NMJ morphology as a result of resistance exercise training were independent of changes in muscle fiber size. Rosenheimer and Smith (1985) found increases in motor endplate size with aging despite decreases in muscle fiber cross-sectional area. It has also been shown that NMJ morphology is related to muscle fiber type (slow vs. fast) (Prakash & Sieck, 1998). We did not visualize different muscle fiber types in this study, but other studies in our laboratory have shown that both before and after stimulation the muscle fiber type of the genioglossus is relatively homogeneous, with approximately 95% or more of the muscle fiber type being type II (unpublished data).

### 2.4.3 Clinical Implications

Current clinical practice using NMES for the treatment of dysphagia uses surface electrodes, not direct nerve stimulation (Clark, 2003). However, because swallowing involves a complex temporal interaction of multiple cranial muscles, treatment of dysphagia with surface electrodes may provide limited benefit (Clark et al., 2009). Furthermore, only muscles directly underneath surface electrodes are stimulated by surface NMES; the deep muscles of the tongue are unlikely to be stimulated by surface NMES (Ludlow et al., 2007). In physical therapy, NMES in conjunction with voluntary exercise has been shown to be more effective than exercise alone to strengthen muscles, but not to develop coordinated activity (Paillard, 2008).
Depending on the underlying cause of the swallowing deficit, therefore, direct nerve simulation may be a better approach, although it is, of course, more invasive. If tongue weakness is contributing to age-related dysphagia, stimulation of the tongue muscles through hypoglossal nerve stimulation rather than surface NMES may be a more effective way to increase strength. Preliminary studies of direct hypoglossal nerve stimulation in humans have suggested that it is a safe and effective treatment for sleep apnea (Kezirian et al., 2010). Our study used direct nerve stimulation to ensure that the tongue muscles were being stimulated. The morphologic changes found in this study suggest improved synaptic efficiency of old stimulated NMJs, which, in turn, may contribute to increased strength and decreased fatigue in the tongue muscles. Based on human studies from the sleep apnea literature and the results of this study, further investigation of direct nerve stimulation for the treatment of dysphagia is recommended.

### 2.4.4 Relationship between Two- and Three-Dimensional Measures

Changes observed in NMJ morphology associated with aging and chronic electrical stimulation depend on the method of measurement. The wide range of $R^2$ values found in the comparisons of two- and three-dimensional measurements, as well as the critical differences in the outcomes of the statistical analyses performed with the two- and three-dimensional measurements in this and other studies (T. Suzuki et al., 2009), indicate the relationship between two- and three-dimensional measurements is inconsistent and unreliable.
There are two potential problems with measuring in only two dimensions. First, by not including the third dimension of depth, NMJs that appear similar in two-dimensional area may actually have different depths and, consequently, have very different sizes. An example of this from our data is the disparity in volume found for 5 endplates with the same area of 170 \( \mu m^2 \), but with volumes of 243, 314, 353, 436, and 670 \( \mu m^3 \). The second potential problem of measuring in two dimensions is the orientation-dependence of two-dimensional measurements. Area and other two-dimensional morphologic measures may vary depending on a structure’s orientation in relation to the image plane, whereas three-dimensional measurements are consistent despite changes in orientation. Figure 2.6 shows z-projections of the same motor endplate in the orientation collected at the microscope, and after being reconstructed in three dimensions and rotated 90°. The area of the motor endplate in the original orientation is 75 \( \mu m^2 \), whereas the rotated endplate has an area of 133 \( \mu m^2 \). Based on these potential problems of two-dimensional measurements, as well as the inconsistency of the relationship between two- and three-dimensional measures found in this study, we conclude that three-dimensional measurements more accurately and consistently capture the size of NMJ morphology.
Contrary to previous studies of NMJ morphology, both from our laboratory (Connor et al., 2002; Hodges et al., 2004) and elsewhere (Balice-Gordon, 1997a), we did not observe significant signs of denervation/reinnervation, such as axonal sprouting or Schwann cell processes. This may have been due to our use of three-dimensional renderings to examine these possible signs. Similar to the orientation-dependence seen with size measurements, the angle at which areas of green and blue staining were observed had an effect on the judgment of these signs. Often an area of staining appeared as a possible Schwann cell process or axonal sprout in the original orientation, but it was subsequently seen to be disassociated from the NMJ when the image was rotated to another angle. Therefore, in this study, age-related changes in NMJ morphology were not consistent with characteristics of the denervation/reinnervation process.
Also, contrary to previous studies in other muscles, we did not find significant effects of age or NMES on nerve terminal size (Connor et al., 2002; Elkerdany & Fahim, 1993; Fahim & Robbins, 1982). This is consistent, however, with previous work in the genioglossus that did not find any significant differences in nerve terminal area between young and old animals (Hodges et al., 2004). Other pre-synaptic parameters have been observed to change with aging, such as increased nerve terminal branching (Elkerdany & Fahim, 1993), and these parameters may provide further insight into age-related changes of NMJs in the tongue.

In conclusion, we have shown that NMES reduced age-related changes in NMJ morphology in an extrinsic tongue muscle in the rat by restoring the relationship between pre- and post-synaptic volumes. Furthermore, significant differences were found between two- and three-dimensional measurements of NMJ morphology. Based on the results of this study, we postulate that functional improvements reported with NMES may be due in part to improved synaptic efficiency of the NMJ. Furthermore, we recommend the use of three-dimensional measurements to quantify changes in NMJ morphology.

**Acknowledgements**

This study was supported by grants from the National Institute on Deafness and Other Communication Disorders (Grants R01DC005935 and R01DC008149). The authors are grateful to Allison Schaser and David Barnett for their assistance in the completion of this work, as well as Lance Rodenkirch and the W.M. Keck Laboratory for
Biological Imaging at the University of Wisconsin–Madison for assistance with and use of the confocal microscope. Dr. Glen E. Leverson performed the statistical analyses, David L. Zealear designed the implants used in the stimulation animals, and Michelle Jackson performed the animal surgery.
Chapter 3: Effects of vocal training on rat ultrasonic vocalizations and neuromuscular junction morphology in the aging rat larynx (study B)

3.1 Introduction

3.1.1 Background and significance

Chronic dysphonia is common in the elderly and has a significant negative impact on quality of life (Golub et al., 2006; Roy, Stemple, Merrill, & Thomas, 2007b; Turley & Cohen, 2009; Verdonck-de Leeuw & Mahieu, 2004). There are well-documented acoustic, aerodynamic, and laryngeal changes in patients with age-related dysphonia (Bloch & Behrman, 2001; Ferrand, 2002; Gorham-Rowan & Laures-Gore, 2006; Hagen et al., 1996; Harnsberger et al., 2009; Hodges et al., 2004). Changes in the senescent larynx that likely contribute to age-related dysphonia include alterations in the biomechanical properties of the vocal folds (Chan & Titze, 1999; Gray et al., 2000; S. Hirano et al., 2004), muscle fiber type conversion from fast to slow fibers in the laryngeal muscles (Rodeno et al., 1993; T. Sato & Tauchi, 1982; T. Suzuki et al., 2002), and denervation-like changes in the laryngeal neuromuscular system (Mortelliti et al., 1990; Perie et al., 1997).

These denervation-like changes are evidenced at the neuromuscular junction (NMJ), which changes in size, complexity, density, and abundance in aging laryngeal muscles (Connor et al., 2002; Gambino et al., 1990; McMullen & Andrade, 2009). Morphologic and physiologic changes in the aging NMJ, including a deterioration of the
pre-post synaptic relationship (Johnson & Connor, 2011), likely lead to reduced synaptic efficiency (Elkerdany & Fahim, 1993; Fahim & Robbins, 1982; Rosenheimer, 1990), and precede atrophic changes in aging muscles (Deschenes et al., 2010). Neural agrin is a proteoglycan in the synaptic basal lamina that is necessary for long-term maintenance of motor endplates (Lin et al., 2001), and is critical for guiding reinnervation (Bezakova, Helm, Francolini, & Lomo, 2001). However, effects of aging on agrin and synaptic maintenance are unknown. Therefore, study of NMJ morphology and its underlying adaptive mechanisms, such as neural agrin, will give us insight into how aging affects the laryngeal muscles and their consequent function and how we can reduce or prevent the effects of aging.

Exercise in the limb musculature has been shown to reduce the effects of age on both muscle function (Fiatarone et al., 1994; Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988; Pette & Staron, 2001), and NMJ morphology and physiology (Alshuaib & Fahim, 1990; Andonian & Fahim, 1988). Exercise programs have been developed for the aging voice, but there is little biological evidence to support the use of these interventions. The effects of vocal exercise on the underlying neuromuscular mechanisms of the larynx are unknown and difficult to study in humans. However, an animal model may prove useful in this regard. Understanding the mechanisms of exercise on the muscles of the upper airway is critical to developing treatments to prevent and/or reverse the effects of age-related voice disorders (Thomas et al., 2008).
The ultrasonic vocalizations (USV) of rats have been used to study a variety of correlates between the brain and behavior, including the central mechanisms underlying laughter and joy (Panksepp, 2007), and vocal deficits related to Parkinson disease (Ciucci et al., 2007; Ciucci et al., 2008). Additionally, acoustic parameters of USVs, including bandwidth, peak intensity, and peak frequency, decrease in aging rats (Basken et al., 2012). Because the rat larynx has been implicated as the source of USVs (Johnson et al., 2010; Riede, 2011; Roberts, 1975a), rat USVs also provide an opportunity to study peripheral neuromuscular changes in response to interventions.

### 3.1.2 Hypothesis

The hypothesis of this study was that age-related changes in ultrasonic vocalizations and laryngeal neuromuscular junctions of senescent rats can be reversed through vocal exercise in the form of vocal training. This hypothesis was tested by comparing USV acoustics, NMJ morphology, and agrin localization in laryngeal muscles in young adult and old rats that were vocally trained versus control groups of animals that were not trained.

### 3.1.3 Specific aims

There were 3 specific aims of this research.

Aim 1: To determine the effect of age on USV acoustics and laryngeal NMJ morphology. The USVs of young adult and old rats were recorded and acoustically
analyzed before the start of an 8-week vocal training program to assess baseline differences between age groups. Changes in NMJ morphology between age groups was assessed by examining NMJ morphology and neural agrin localization in the thyroarytenoid muscles in a control group of rats that did not receive vocal training.

Aim 2: To determine the effect of vocal training on USV acoustics and laryngeal NMJ morphology. Both young adult and old rats were divided into vocal exercise and control groups. After 8 weeks of either the vocal training or a control condition, USVs, NMJ morphology, and neural agrin localization in the lateral and medial thyroarytenoid muscles were compared to assess the effects of vocal training and any interaction with age.

Aim 3: To determine the relationships between age, training, USV acoustics and laryngeal NMJ morphology. Analyses were conducted to determine if changes in USV acoustics correlated with changes in measures of NMJ morphology.
3.2 Methodology

3.2.1 Experimental overview

Overall procedure

The overall goal of this study was to examine how age and vocal exercise modify the ultrasonic vocalizations (USVs) of rats and the neuromuscular mechanisms that may underlie those vocalizations. The first step toward this goal was to assess the effects of age on USVs by comparing baseline (pre-intervention) USVs from young adult and elderly rats. Next, the two age groups of rats were equally and randomly assigned to either an intervention (vocal exercise) group or a control group that did not receive vocal exercise. After 8 weeks of either the control condition or vocal exercise, USVs were again compared to assess the effects of vocal exercise and any interaction between vocal exercise and age. Lastly, laryngeal neuromuscular junctions were examined using immunohistochemistry and confocal microscopy.

Animals

A total of 43 Fischer 344/Brown Norway (F344BN) male rats, 20 young adult and 23 old, were obtained from the National Institute on Aging animal colony. This inbred strain of rat is used frequently in aging research because rats are genetically identical, thus reducing individual phenotypic variation. Young adult rats were 9 months old, representing mature young adulthood, and elderly rats were 32 months old, representing advanced senescence. The median lifespan for this strain of rats is 33 months (Turturro et al., 1999). Only male rats were used because the female estrus cycle affects USV
production (Matochik, White, & Barfield, 1992). An equal number of rats from each age group were randomly assigned to either an intervention (vocal exercise) group or a control group. Three rats in the old group died from natural causes, leaving an equal number of rats (10) in each age/experimental group combination.

Animal conditions

All rats were kept on a reversed light cycle because rats are nocturnal animals. This allowed for training during their active time while allowing the investigator to work during typical daytime hours. All investigator-rat interactions took place in an isolated, darkened room dimly illuminated with red light. Rats remained in their home cages for all recordings, vocalization training sessions, and control condition interactions. Water and food were provided ad libitum. Rats were singly housed, because socially isolated rats have a more robust response to methods that elicit vocalizations than do socially housed rats (Panksepp & Burgdorf, 2000).

3.2.2 Behavioral training

Baseline and post-training USV recordings

Equipment

USVs were monitored and recorded using an ultrasonic microphone and USB recording interface connected to a Windows PC running Avisoft-RECORDEr (Avisoft Bioacoustics, Berlin, Germany). The ultrasonic condenser microphone (UltraSoundGate CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) had a frequency range of 10 - 200
kHz and a flat frequency response between 30 - 65 kHz. The ultrasound recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany) supplied the microphone with the required 200 V polarization voltage, converted the analog acoustic signal to a 16-bit digital signal, and sent this digital signal to the computer via USB 2.0. The Avisoft-RECORDER software generated a real-time spectrogram used to monitor USVs during baseline and post-training recordings, as well as during the 8-week vocal exercise training.

Procedure

Baseline and post-training USV recordings from all rats were obtained using an existing procedure that reliably elicits vocalizations in male rats (Ciucci et al., 2008; Johnson et al., 2011). The procedure involved pairing a male rat with a sexually receptive female rat in estrus. Behavioral signs exhibited by a female rat in estrus include lordosis (hunching the back), darting around the cage, and rapid ear wiggling (Nelson, 2005). Once the male responded to the female by sniffing, chasing, and/or mounting, the female was removed, eliciting vocalizations from the male. Vocalizations from each male were recorded and counted for 1 minute. If a minimum of 20 vocalizations had not been recorded after 1 minute, recordings continued until at least 20 vocalizations were obtained. One audio file per rat was saved for later offline acoustic analysis.
**Vocal Training**

**Duration**

The 20 rats assigned to the vocal exercise group were trained 5 days a week for 8 weeks to progressively increase their production of 50-kHz USVs. An 8-week training period was chosen for two reasons. First, clinical vocal exercise programs typically range in length from 4 to 8 weeks (Spielman et al., 2007; Stemple et al., 1994; Stemple et al., 2009). Secondly, changes in NMJ morphology have been reported after similar lengths of progressive exercise programs in the limb musculature (Deschenes et al., 2000), and chronic nerve stimulation in the tongue musculature (Johnson & Connor, 2011).

**Training protocol**

Vocal exercise sessions took place in the same environment as the baseline and post-training USV recordings. Initially, vocalizations were elicited through interaction with a female rat as described above. However, rats also produce 50-kHz calls in anticipation of a positive reward (Burgdorf et al., 2008). Consequently, an operant conditioning paradigm was used in which male rats were rewarded with a food treat when they began vocalizing in response to the female. After 2-4 weeks, depending on the individual rat, males vocalized in anticipation of the food reward and the female stimulus was no longer needed, or was only needed sporadically. At first, any production of a 50-kHz vocalization was rewarded. As training progressed, rewards were faded and given after every 5 vocalizations or after a string of vocalizations in rapid succession. Vocalizations were visually and aurally monitored and manually counted during each
session. Each training session lasted until the rat produced a target number of vocalizations.

Session Criteria

Although initial vocalization rates varied among rats, all rats in the vocal exercise group were trained to produce the same number of vocalizations in each session to equalize the exercise dose. The initial target of 50 vocalizations per session was determined by calculating the first quartile of the number of vocalizations produced during the baseline recordings (47). An initial target easily achieved by the majority (75%) of rats was selected to provide an acclimation period for the operant conditioning. After the first 2 weeks of training, the target number was increased according to the following schedule: week 3 (75), week 4 (100), week 5 (150), week 6 (200), week 7 (250), week 8 (300).

Control Group

Rats in the control group were treated identically to the vocal exercise group, but did not receive vocal exercise. Control animals were housed in the same room as the vocal exercise group in the animal care facility for 8 weeks. In lieu of the vocal exercise program, the control animals were hand-fed food treats in their home cage 5 days a week to control for social effects of human contact. To ensure this interaction did not elicit USVs, the control interaction was monitored once a week using the same ultrasonic recording setup used for vocal exercise training.
3.2.3 USV analysis

Baseline and post-training USVs were analyzed using SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) following an established protocol (Johnson et al., 2011). After creating a spectrogram of the sound file, the beginning and end of each 50-kHz USVs were automatically defined using an intensity threshold. Interfering noise and 22-kHz USVs were manually erased from the spectrogram. Next, each vocalization was classified by an experienced rater and labeled into one of 4 categories (Figure 3.1) based on visual inspection of its frequency characteristics: flat (steady frequency), frequency modulated (FM) (regular frequency modulation with a sine wave-like appearance), harmonic (fundamental frequency near 30 kHz with a visible harmonic one octave above), or step (abruptly bifurcated frequency components). These categories of 50-kHz USVs are well-established, have been shown to respond differently to age and disease, and have strong inter- and intra-rater reliability (Basken et al., 2012; Ciucci et al., 2009). The age and experimental groups of the recordings were masked during classifications. The acoustic parameters of all labeled vocalizations were then automatically measured by SASLab Pro. USVs identified as either FM, harmonic, or step were grouped together for statistical analyses because of their relative acoustical complexity (“complex USVs”), compared with the acoustically simple flat USVs (“simple USVs”).
Figure 3.1. Spectrogram of representative USVs from the 4 classifications: (A) & (B) simple (short & long durations), (C) & (D) frequency modulated (FM) (small & large bandwidths), (E) harmonic, and (F) step. For acoustic analysis, FM, harmonic, and step USVs analyzed together as “complex”.
3.2.4 Tissue collection and immunohistochemistry

Tissue collection

After the post-training recordings, rats were euthanized via an IP injection overdose of Beuthanasia. The entire larynx was immediately dissected and fixed for 1 hour in 4% formaldehyde. After fixation, each larynx was washed 3 x 5 min and submersed overnight in a mixture of 20% sucrose and 5% glycerol in phosphate buffered saline to reduce ice crystal formation during flash-freezing. After cryoprotecting, each larynx was flash frozen in isopentane chilled to just above its freezing point in liquid nitrogen and individually stored in a -80 degree Celsius freezer.

Tissue sectioning

Using a cryostat set to -20 degree Celsius, larynges were sectioned in the transverse plane until both the lateral and medial thyroarytenoid (TA) muscles were visible. The presence of both the medial and lateral TA muscles was confirmed by viewing the sections through a transmitted-light microscope to ensure the muscles extended from the arytenoid cartilage to the interior of the thyroid cartilage. Eight 30-µm thick sections that included both the medial and lateral thyroarytenoid (TA) muscles were alternately mounted on two slides, 4 sections per slide. One slide was used for NMJ morphology measures and the other was used to assess neural agrin localization. The 40 larynges were sectioned and stained in 8 groups of 5, with a mix of age and experimental condition in each group. Immediately after sectioning, slides were immersed in 1x tris
buffered saline (TBS) and mildly agitated at room temperature until all 5 larynges in the group were sectioned. All slides were then washed for 5 min in 1x TBS.

**Immunohistochemistry**

To label NMJs with fluorescent antibodies, sections were first washed with 0.3% Triton X-100 in TBS 2 x 5 min to improve antibody penetration. This was followed by a 90-minute incubation in blocking solution containing 5% normal goat serum and 1% bovine serum albumin in TBS to reduce secondary antibody cross-reaction with endogenous immunoglobulins.

The three primary structures of the NMJ (terminal Schwann cells, nerve terminal, and motor endplate) along with the innervating axon were labeled using primary antibodies for each target followed by fluorescent secondary antibodies (Figure 3.2) (Balice-Gordon, 1997a; Fox, Ho, Smyth, & Sanes, 2008; Johnson & Connor, 2011). For neural agrin localization, both neural agrin and the motor endplates were labeled (Stone & Nikolics, 1995; T. Suzuki et al., 2008).

Sections were incubated overnight in a humidified chamber at 4° Celsius in the following primary antibodies diluted in wash buffer: α-bungarotoxin conjugated Alexa488 diluted at 1:1000 to label acetylcholine receptors in the motor endplate (Invitrogen: Molecular Probes, Eugene, WA); Znp1, mouse/IgG2a, diluted at 1:200 to label synaptotagmin in the nerve terminal membrane (Zebrafish International Resource
Center, Eugene, OR); anti-S100β, rabbit/polyclonal, diluted at 1:1000 to label myelinating and terminal Schwann cells (Invitrogen: Molecular Probes, Eugene, WA); and neurofilament-M, chicken/polyclonal, diluted at 1:1000 to label neurofilaments of the axon (antibodies-online GmbH, Atlanta, GA). Sections stained for neural agrin were incubated in the same dilution of α-bungarotoxin conjugate plus Agr-131, mouse/IgG2a, diluted at 1:500 to label agrin (Enzo Life Sciences, Plymouth Meeting, PA).

The next day, sections were washed 3 x 5 min in blocking buffer and incubated for 4 hours at room temperature in following fluorescent secondary antibodies diluted in wash buffer: Alexa568, goat/anti-mouse IgG2a, diluted at 1:500 (Invitrogen: Molecular Probes, Eugene, WA); Alexa633, goat/anti-rabbit IgG, diluted at 1:1000 (Invitrogen: Molecular Probes, Eugene, WA); and DyLight 549, goat/anti-Chicken IgY, diluted at 1:2000 (Jackson ImmunoResearch, West Grove, PA). Sections stained for neural agrin were incubated in Alexa568, goat/anti-mouse IgG2a, diluted at 1:500 (Invitrogen: Molecular Probes, Eugene, WA).

After secondary antibody incubation, sections were washed 3 x 5 min in blocking buffer, cleared with Histoclear (National Diagnostics, Atlanta, GA), and covered with a #1.5 coverslip using a polyvinyl alcohol based mounting media. At the conclusion of staining, slides were coded by a third party to mask age and experimental group during microscopy and image analysis.
Figure 3.2. Photomicrographs of maximum z-projections from unmixed spectral confocal microscope image stacks demonstrating the 4 structures in the NMJ. (lateral TA from a rat in the old trained group) (scale bar = 5 µm).
3.2.5 NMJ morphology imaging and analysis

*Spectral confocal microscopy*

NMJs were imaged using a spectral confocal microscope (A1R, Nikon, Melville, NY) at the W.M. Keck Laboratory for Biological Imaging at the University of Wisconsin-Madison. In traditional multi-fluorescent label (non-spectral) confocal microscopy, the fluorescent emission is divided into discrete bandwidths centered approximately on the emission peak of each fluorescent label. The light is then collected by a single-channel photon multiplier tube (PMT), requiring multiple scans and/or multiple PMTs to capture the emission from each fluorescent label.

There are two primary limitations to this method. First, in addition to collecting the desired emission from the fluorescent label, the bandwidth also includes any endogenous fluorescent emissions from the specimen (autofluorescence) as well as emission from any spectrally neighboring fluorescent labels that might be simultaneously excited (bleed through). This can usually be overcome by adjusting immunohistochemistry parameters and choosing fluorophores with minimally overlapping excitation and emission spectrums. A second limitation of traditional confocal microscopy is that a maximum of 3 fluorophores can typically be used to label structures because of the difficulty in finding a combination of primary antibodies and fluorophores that do not overlap in their reactivity and/or spectra. These two limitations of traditional confocal microscopy are easily overcome by using spectral confocal microscopy (Mansfield, Gossage, Hoyt, & Levenson, 2005).
In spectral confocal microscopy the entire emission spectrum from multiple fluorophores is collected at one time by a multi-channel PMT. After acquiring the multi-channel image, individual fluorophores and background autofluorescence are separated from each other through a post-processing technique known as linear unmixing. This mathematical process combines the known spectra of the fluorophores and background fluorescence, either from a database or single-labeled samples, to unmix the signals. This allows for labeling samples with multiple, overlapping fluorophores that can be simultaneously excited and collected. The primary disadvantages of spectral confocal microscopy are the additional time and computational resources needed for the post-acquisition unmixing.

**Equipment**

In this study, three laser lines (488, 562, and 638 nanometers (nm)) simultaneously excited the four fluorophores labeling the axons, nerve terminals, Schwann cells, and motor endplates. Prior to collecting images, it was empirically determined that laser powers of 20, 3, and 25 for lines 488, 562, and 638 respectively, and a PMT high voltage of 150 achieved maximum signal without fluorophore saturation. A 32-channel PMT collected the emission spectrum from 502-694 nm in 6 nm bins. These settings were used throughout image collection.
Image Resolution

The resolution of a laser scanning confocal microscope is determined by the amount of light that is passed to the detector (PMT). This, in turn, is controlled by: 1) the numerical aperture of the objective lens and 2) the size of the pinhole. For this experiment, a 60x water-immersion Plan Apo VC objective with a 0.27mm working distance and a 1.2 numerical aperture was used. The confocal pinhole was opened to 43.2 μm, which was 1 Airy Unit for the longest wavelength laser (637 nm). This objective/pinhole combination created a lateral (xy) resolution of 150 nm and an optical sectioning (z) resolution of 880 nm. In other words, 2 points of light closer to each other than the distances above would have appeared as a single point of light in the collected image. Conversely, 2 points farther apart than these distances would have appeared as distinct points. These resolutions were more than adequate to capture, reconstruct, and measure three-dimensional NMJ morphology.

Image size

In addition to the 60x magnification of the objective lens, an additional 1.383x digital zoom was used to create a lateral pixel dimension of 75 nm, half the lateral resolution of the microscope (150 nm). In the z-dimension, image stacks were collected using 300 nm steps, approximately one-third of the optical sectioning resolution. The 1024x1024 scan area was cropped to include 1-3 NMJs per scan, reducing both the scan time and image file size. The scan speed was 1/4 frame/sec with a pixel dwell time of 2.4 microseconds.
Collection of NMJs

NMJs were collected from both the left and right sides of at least 3 different laryngeal sections to account for possible intra-muscle variability. The location of each image was recorded in a spreadsheet and encoded into the image name. A minimum of 18 NMJs per muscle (medial and lateral TA) were collected, for total of 36 NMJs for each animal. Previous studies examining the effects of age and neuromuscular activity on NMJ morphology have found differences using sample sizes ranging from 10 to 30 NMJs per muscle (Connor et al., 2002; Deschenes & Wilson, 2003; Deschenes et al., 2006; Johnson & Connor, 2011; Kawabuchi et al., 2001; Wang et al., 2004). To ensure that each NMJ was completely contained in the z-series image stack, the top and bottom of the tissue section were located by focusing through the section until reflections from the cover slip and slide were observed. Only NMJs that were entirely within these upper and lower bounds with a margin of at least four z-planes (1.2 µm) were captured. The use of these margins ensured that entire NMJs with full pre and post synaptic components were captured.

Qualitative image analysis

NMJ morphology was assessed both qualitatively and quantitatively. All measurements were made in three dimensions, as area measurements of two-dimensional projections are less accurate than volumetric measurements of three-dimensional reconstructions. (Johnson & Connor, 2011; Prakash, Smithson, & Sieck, 1993; Wang et al., 2004). Using reference spectra collected from single-labeled laryngeal specimens, the
32-channel spectral images were linearly unmixed to create 4-channel images, one channel for each fluorescent label.

Qualitative measures of the presence/absence of 1) axon withdrawal, 2) terminal Schwann cell projection(s), and 3) extra-junctional axon sprout(s) were then made by examining both the 4-color three-dimensional reconstruction in Elements AR (Nikon) and each single-color channel in grayscale to avoid color bias (Figure 3.3). Axon withdrawal was assessed by the presence/absence of a stained axon in contact with the endplate. Schwann cell projections and extra-junctional axon sprouts were positively identified as a narrow, finger-like extension of staining beginning from and extending beyond the NMJ. After completing the qualitative measures, individual NMJs were cropped in 3D and saved in 2-color image stacks with the endplate and nerve terminal staining.

One year after all qualitative measures had been completed, approximately 5% (80) of the total number of NMJs were selected to measure inter-rater and intra-rater reliability. Sixty images were randomly selected from the larger set of images that had been identified as exhibiting the 3 qualitative measures (20 images for each measure). An additional 20 NMJs were included that did not exhibit any of the 3 qualitative features. A judge external to this study who was familiar with the staining and microscopy procedures (AJS) but naïve to these particular images made independent judgments on the 3 qualitative measures. The same judge who had performed the initial measurement
of the full data set (AMJ) also judged the 5% subset of images. Inter- and intra-rater reliability (% agreement) were both between 82-89% (Inter-rater: axon withdrawal, 86.2%; Schwann cell processes, 82.5%; axon sprouts, 87.5%. Intra-rater reliability: axon withdrawal, 88.8%; Schwann cell processes, 83.8%; axon sprouts, 85.0%).
Figure 3.3. Maximum z-projections of confocal image stacks showing examples of (A) an NMJ with no qualitative signs of pre-synaptic remodeling from an old trained rat, (B) axon withdrawal from 2 motor endplates from a young trained rat (note axon bundle on top right but no axon extending to either motor endplate), (C) a Schwann cell projection (arrowhead) from an NMJ from an old trained rat, and (D) an axon sprout (arrowhead) from an NMJ from an old control rat (scale bars = 5 µm).
Quantitative image analysis

Images were quantitatively measured in ImageJ (Abramoff, Magelhaes, & Ram, 2004) using an automated measurement algorithm adapted from study A.

Preprocessing

The 2-color image stacks were imported into ImageJ as separate single-color stacks. To reduce noise a 3x3 2D median filter with a 2 pixel radius was applied to each slice. To account for inherent variations in intensity levels common with immunohistochemistry and confocal microscopy, the dynamic intensity range of motor endplate stacks was normalized by linearly scaling the 12-bit images to 8-bit so that the minimum to maximum range of intensities in each image was 0 to 255. Motor endplate image stacks were then converted to binary by applying an absolute threshold of 10, meaning all pixels with an intensity of 10 or greater were considered to be part of the foreground, while any pixel with an intensity level less than 10 was background. Nerve terminal 12-bit image stacks were also converted to binary using an intensity threshold of 10. This intensity was chosen as the threshold because the spectral unmixing process resulted in highly specific images with little background. Binary image stacks were furthered processed to reduce noise and remove small, isolated structures by applying a 3D median filter with a 3 pixel radius followed by a 3D erosion and dilation.
Volume

Volumes (μm$^3$) of the binary nerve terminal and motor endplate stacks were measured by multiplying the stained area on each slice by the stack spacing (0.3 μm).

Synaptic overlap

Synaptic overlap was defined as the percentage of the total stained endplate area that was overlapped by the combined endplate and terminal stained area on a maximum z-projection (Figure 3.4). This overlap should be greatest when viewing the synapse en face, since the NMJ lies relatively flat along the muscle fiber. Because it was not possible to rotate the NMJ to an en face orientation during imaging, endplate image stacks were rotated to an approximated en face orientation. This orientation was found by rotating the NMJ until the maximum area on a z-projection was found. This was done first in the motor endplate stack by measuring the area of the maximum z-projection as each endplate stack was rotated in 1° increments on the y-axis. After the maximum area on the y-axis was found, the stack was rotated to that y-axis position and the procedure was repeated for the x-axis. The corresponding nerve terminal image stack was then rotated on its y and x axes by the same degree as the endplate stack. All stacks were rotated using the ImageJ plug-in TransformJ (Meijering, Niessen, & Viergever, 2001).
Figure 3.4. Two images of the same NMJ demonstrating rotation of motor endplate (red) and nerve terminal (green) to an *en face* orientation to calculate synaptic overlap (yellow). (A) Original orientation collected at the microscope. The nerve terminal appears to be slightly to the left of the endplate, indicating the NMJ was imaged on its side. In this orientation, 82% of the motor endplate is overlapped by the nerve terminal. (B) View after rotating both the terminal and endplate 140° on the x-axis and 103° on the y-axis. These degrees of rotation give the maximum projected area for this particular endplate, providing an *en face* view. The nerve terminal now overlaps 98% of the motor endplate.

Motor endplate dispersion

Motor endplate dispersion volume was defined as the percentage of space occupied by the motor endplate beyond its stained regions. This was calculated by comparing the total occupied volume to the stained volume (occupied volume/stained volume x 100 - 100). Thus, the stained volume was defined as the stained area on each slice multiplied by the stack spacing. The occupied volume was defined as the stained volume plus the space in between stained areas on each slice. This was automatically determined using a convex hull algorithm that enclosed the stained volume within a region of interest without any concavity (Convex hull plus, G. Landini, 2004). A tightly
packed endplate with very little space between the stained area(s) would have a dispersion volume close to 0% (equal stained and occupied volumes). Dispersion volume was calculated after rotating the endplate image stack to a standardized orientation to reduce the influence of microscope orientation. The standardized orientation was found by rotating endplate stacks on the y and x axes to the angles that achieved the smallest two-dimensional convex hull perimeter on a maximum z-projection.

Motor endplate dispersion is most commonly reported as a two-dimensional measure obtained using flattened z-series from traditional confocal microscopy (Deschenes et al., 2000). However, two-dimensional measurements of NMJ morphology obtained in this manner are affected by the angle at which the NMJ resides in the tissue relative to the microscope plane or angle of view (Johnson & Connor, 2011). That is, NMJ components that are not en face with respect to the microscope plane will not be accurately measured using two dimensional imaging and measurement (Johnson & Connor, 2011). Thus, to obtain a more accurate measure of en face motor endplate area and dispersion, three dimensional image processing methods were used to rotate the motor endplate into an en face orientation (as described above for synaptic overlap) prior to obtaining the area measurement. Stained motor endplate area and convex hull area were measured on a maximum z-projection of the rotated stack. En face dispersion was then calculated using a similar algorithm as described above for dispersion volume (en face dispersion = convex hull area/stained area x 100 - 100).
**Fragmented endplates**

The number of motor endplate fragments was defined as the number of discrete three-dimensional objects in each binary endplate image stack. These objects were automatically counted using the 3D Object Counter Image plugin (Bolte & Cordelieres, 2006). An endplate was scored as fragmented if it contained 2 or more 3D objects.

### 3.2.6 Agrin imaging and analysis

**Confocal microscopy**

Traditional confocal microscopy was used to image the double-labeled endplate and agrin muscle sections. The same confocal microscope described above was used with the same 60x water immersion lens. Two laser excitations (488 and 561 nm) sequentially excited the 2 fluorophores. The goal of this measurement was to identify the presence or absence of agrin at the motor endplate and not to precisely calculate 3D spatial measurements. Therefore, images were collected at a lower resolution by opening the pinhole to 1.3 Airy units and without a digital zoom. This resulted in a lateral (xy) resolution of 0.13 µm and an optical sectioning resolution (z) of 0.87 µm. The digital image resolution was 512x512 pixels and the scan speed was 1/2 frame/sec with a resulting pixel dwell time of 4.5 µs. Image stacks were collected with a z-step equal to the optical resolution (0.87 µm). This captured all the fluorescent data in the z-plane without oversampling. At least 30 NMJs per muscle per animal were collected. As with the NMJ morphology image collection, agrin images were collected from both the left and right side of the larynx and from at least 3 different muscle sections.
Image processing

The presence or absence of agrin in individual NMJs in each agrin/motor endplate image stack was automatically determined through algorithmic processing and analyzing with ImageJ (Abramoff et al., 2004; T. Suzuki et al., 2008). First, 12-bit image stacks were split into two single-color stacks. Each stack was then converted to a binary stack by applying an intensity threshold of 250 to the agrin images and 700 to the endplate images. These values were empirically determined by examining a subset of images (10%) and adjusting the threshold until foreground objects were clearly delineated from the background. Individual motor endplate regions were automatically defined on a maximum z-projection using the analyze particles plugin. Presence of agrin was positively identified at each endplate if there was overlap between the binary endplate and agrin image stacks within each NMJ region of interest (Figure 3.5).
Figure 3.5. Sample image from agrin analysis. Z-projection of a 2-color confocal image stack after it was converted to binary using an intensity threshold (light blue = agrin, red = motor endplate, white = overlap). Image is from a rat in the young trained group. (scale bar = 10 µm).
3.2.7 Statistical analyses

Each dependent variable was tested for equality of variances between combined age and experimental groups using Levene's test. Based on variance equality, either the Student's or Welch's t-test was used to test for differences between age groups in baseline USV measurements.

To analyze post-training differences in both USV and NMJ measurements, 2-way Analysis of Variance (ANOVA) tests were used examining main effects of age and training as well as their interaction. In the case of unequal variances between groups, a log transformation was attempted. If heteroskedasticity persisted, the non-parametric Kruskal Wallis Test One Way ANOVA by Ranks was used. Post-hoc testing was completed using the Fisher’s protected least significant difference (LSD) test or Wilcoxon rank sum test in the case of heteroskedasticity.

Effect sizes (Hedges' unbiased d) with 50% and 95% confidence intervals were calculated to further explore the relationships between old and young adult groups within the trained and control groups (Nakagawa & Cuthill, 2007). The Pearson product-moment correlation coefficient was calculated for all post-training variables (USV acoustics, NMJ morphology, and agrin localization) to explore relationships between changes in vocalizations and underlying neuromuscular mechanisms.
For the variables that did not pass Levene's test for equal variances, further testing was performed to determine if age and/or training affected the variance between groups. It was hypothesized that training would reduce measurement variability.

The experimental unit was the individual rat. Therefore, multiple measures from each rat were averaged for each dependent variable to arrive at a single representative measure. This accounted for variance within each animal. All statistical analyses and graphs were completed using R. (R Development Core Team, 2012; Morales, 2011; Sarkar, 2008).
3.3 Results

3.3.1 USV

Baseline equality of experimental groups

Comparison of the trained and control groups at baseline revealed 1 out of the 10 measures of USV acoustics was significantly different. The duration of simple USVs in the control group was shorter than in the trained group ($F_{1,36}=4.36$, $p=0.04$). Therefore, further analysis of the duration of simple USVs was not performed.

Effects of age

Baseline

Young adult and old groups differed in the duration, amplitude, and frequency of their USVs at baseline (Table 1) (Figure 3.6). The complex USVs of the old group were significantly longer in duration than the complex USVs of the young adult group ($F_{1,36}=6.32$, $p=0.02$). Maximum amplitude in the young adult group was greater than in the old group for both simple ($F_{1,36}=18.40$, $p=0.02$) and complex USVs ($F_{1,36}=8.84$, $p=0.03$). The young adult group had a higher mean frequency of simple USVs than old ($F_{1,36}=5.68$, $p=0.02$); the frequency of complex USVs was not significantly different between groups ($F_{1,36}=2.96$, $p=0.09$). There were no baseline age differences in vocalization rate, the percentage of complex USVs, FM bandwidth or maximum frequency slope.
Table 1. Main effects of age and training on USVs

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th></th>
<th>After 8 Weeks</th>
<th></th>
<th></th>
<th>Control</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td></td>
<td>Young</td>
<td>Old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vocalization rate (per minute)</td>
<td>35.5 ± 5.1</td>
<td>31.4 ± 3.5</td>
<td></td>
<td>36.6 ± 3.7</td>
<td>31.6 ± 4.9</td>
<td>23.8 ± 3.4</td>
<td>44.4 ± 4.0</td>
</tr>
<tr>
<td>Percentage complex (%)</td>
<td>65.6 ± 2.2</td>
<td>70.0 ± 2.8</td>
<td></td>
<td>67.7 ± 2.0</td>
<td>61.7 ± 3.0</td>
<td>62.8 ± 2.7</td>
<td>66.6 ± 2.4</td>
</tr>
<tr>
<td>Duration simple (ms)</td>
<td>16.6 ± 1.2</td>
<td>18.6 ± 1.4</td>
<td></td>
<td>19.1 ± 1.3</td>
<td>19.5 ± 1.4</td>
<td>17.8 ± 1.3</td>
<td>20.8 ± 1.4</td>
</tr>
<tr>
<td>Duration complex (ms)</td>
<td>29.6 ± 0.9</td>
<td>32.9 ± 0.9</td>
<td>a</td>
<td>28.4 ± 0.8</td>
<td>37.9 ± 1.7</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Maximum amplitude simple (dB)</td>
<td>-40.0 ± 1.1</td>
<td>-43.7 ± 1.0</td>
<td>a</td>
<td>-44.5 ± 0.5</td>
<td>-47.5 ± 0.5</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Maximum amplitude complex (dB)</td>
<td>-35.9 ± 0.9</td>
<td>-38.9 ± 1.0</td>
<td>a</td>
<td>-40.0 ± 0.6</td>
<td>-42.3 ± 0.5</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>Mean frequency simple (kHz)</td>
<td>54.2 ± 0.6</td>
<td>51.8 ± 0.8</td>
<td>a</td>
<td>58.9 ± 0.7</td>
<td>57.6 ± 0.9</td>
<td>59.3 ± 0.7</td>
<td>57.3 ± 0.9</td>
</tr>
<tr>
<td>Mean frequency complex (kHz)</td>
<td>53.5 ± 0.6</td>
<td>52.0 ± 0.6</td>
<td></td>
<td>60.0 ± 0.8</td>
<td>56.7 ± 0.8</td>
<td>57.9 ± 0.8</td>
<td>58.8 ± 1.0</td>
</tr>
<tr>
<td>Bandwidth FM (kHz)</td>
<td>18.3 ± 0.6</td>
<td>18.9 ± 0.9</td>
<td></td>
<td>18.1 ± 0.8</td>
<td>16.9 ± 0.9</td>
<td>16.4 ± 0.8</td>
<td>18.6 ± 0.8</td>
</tr>
<tr>
<td>Maximum FM frequency slope (kHz/ms)</td>
<td>12.3 ± 0.6</td>
<td>12.0 ± 0.8</td>
<td></td>
<td>10.6 ± 0.6</td>
<td>9.8 ± 0.6</td>
<td>9.8 ± 0.6</td>
<td>10.7 ± 0.6</td>
</tr>
</tbody>
</table>

a Significant difference (p ≤ 0.05) from young group
i Significant interaction (p ≤ 0.05) between age and training

Values are means +/- standard error; n = 40 (10 per age/experimental group combination)
Figure 3.6. Representative USVs recorded post-training demonstrating the smaller amplitude found in USVs from (A) an old control rat, compared with USVs from both (B) an old trained rat, and (C) a young control rat.
Post-training

A significant main effect for age was also present post-training, with a longer duration of complex USVs in the old group than in the young group (control and trained groups combined) \( F_{[1,36]}=29.63, p<0.001 \). The magnitude of this effect increased approximately threefold from baseline.

Post-training age effects were different from baseline age effects. At baseline, frequency of complex USVs in the old group was lower, but not significantly different than the young group. However, post-training, this difference in frequency between young and old groups increased and was significant \( F_{[1,36]}=8.47, p=0.006 \). The baseline significant difference in mean frequency of simple USVs between age groups was not observed post-training \( F_{[1,36]}=1.33, p=0.26 \).

Effects of training

All trained rats in both age groups successfully met the weekly training targets and vocalized 300 times per session by the end of the intervention. Examination of post-training USVs showed training had a significant effect on vocalization rate and impacted USV acoustic parameters, including amplitude, frequency, and duration. These results are discussed in detail below.

There was a significant interaction between training and age on vocalization rate \( F_{[1,36]}=4.53, p=0.04 \). Fisher's protected LSD test showed two significant differences; the
old trained group had a higher vocalization rate than the old control group (p=0.0001), and the young adult control group had a higher vocalization rate than the old control group (p=0.03). Comparing baseline to post-training call rates showed that 14/20 of the trained rats increased their vocalization rate from baseline, whereas the opposite was seen in the control rats; 14/20 of the control rats decreased their vocalization rate after 8 weeks of no training (Figure 3.7).

**Figure 3.7:** Comparison of vocalization rate pre/post intervention. Symbols above the 45° dashed line indicate an increased rate after 8 weeks relative to baseline.
Training reduced the amplitude difference between old and young adult groups seen at baseline. Although there was not a significant interaction between age and training ($F_{1,36} = 2.83$, $p=0.10$ for simple; $F_{1,36}=1.87$, $p=0.18$ for complex), examination of effect sizes showed the post-training difference in amplitude was significant only between young adult and old in the control group (95% confidence interval not overlapping zero) and not in the trained group (Figure 3.8).

**Figure 3.8**: Effect sizes with confidence intervals of comparisons between young and old demonstrating that the young adult control group had higher USV amplitudes than old in control group only (open circle = control group, filled circle = trained group).
Training did not affect USV duration or frequency, although there were age effects that differed from those observed at baseline (discussed above). There were no training effects in the percentage of complex USVs, FM bandwidth, or maximum frequency slope.

3.3.2 NMJ

A total of 1185 images containing 1655 NMJs were collected. During microscopy, NMJs in the lateral and medial TA appeared to differ in volume and distribution (Figure 3.9). Therefore, the dependent variables were compared between the two portions of the muscle using Welch Two Sample t-tests. Results from this testing revealed the lateral and medial TA were different on 7 of 10 variables of NMJ morphology (Table 2). Subsequently, comparisons were made separately within each muscle for all 10 dependent variables.

Variance between groups was unequal on 7 variables. Of these 7 variables, 3 were log-transformed to achieve homoskedasticity (median en face dispersion in the lateral TA and fragmented endplates in both the lateral and medial TA). The remaining 4 homoskedastic variables (axon sprouts in the lateral TA, and axon withdrawal, median dispersion volume, and percentage of agrin overlap in the medial TA) were analyzed using non-parametric testing. Results for all tests of age and training are summarized in Table 3 and Table 4.
Figure 3.9: Micrograph of the distribution of motor endplates in the rat TA. Widefield fluorescent image (4x objective) of a 50-μm thick transverse section from a rat larynx. Acetylcholine receptor clusters in the motor endplates are labeled with Alexa Fluor 488 conjugated α-bungarotoxin. Note the difference in the distribution between the horizontal endplate band in the lateral TA compared with the diffuse distribution along the length of the medial TA (scale bar = 1mm).
Table 2. Differences in NMJs between lateral and medial TA muscles

<table>
<thead>
<tr>
<th>Pre-synaptic remodeling</th>
<th>L-TA</th>
<th>M-TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon withdrawal (%)</td>
<td>40.4 ± 3.4</td>
<td>16.8 ± 2.5 a</td>
</tr>
<tr>
<td>Axon sprouts (%)</td>
<td>5.7 ± 1.3</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>Terminal Schwann cell processes (%)</td>
<td>17.1 ± 2.3</td>
<td>13.2 ± 1.7</td>
</tr>
<tr>
<td>Size &amp; pre/post relationship</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve terminal volume (um3)</td>
<td>75.5 ± 6.4</td>
<td>108.1 ± 7.4 a</td>
</tr>
<tr>
<td>Motor endplate volume (um3)</td>
<td>236.2 ± 6.2</td>
<td>109.1 ± 3.2 a</td>
</tr>
<tr>
<td>Overlap (%)</td>
<td>34.1 ± 3.1</td>
<td>68.0 ± 3.2 a</td>
</tr>
<tr>
<td>Endplate stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median dispersion volume (%)</td>
<td>38.3 ± 0.8</td>
<td>39.6 ± 1.4</td>
</tr>
<tr>
<td>Median en face dispersion (%)</td>
<td>12.2 ± 0.5</td>
<td>10.2 ± 0.5 a</td>
</tr>
<tr>
<td>Fragmented endplates (%)</td>
<td>20.1 ± 2.0</td>
<td>27.0 ± 2.0 a</td>
</tr>
<tr>
<td>Agrin overlap (%)</td>
<td>74.5 ± 4.1</td>
<td>94.8 ± 1.6 a</td>
</tr>
</tbody>
</table>

*a* Significant difference (p ≤ 0.05) Welch Two Sample t-test

n = 40 (20 per muscle)
Table 3. Main effects of age and training on NMJs in the lateral TA

<table>
<thead>
<tr>
<th></th>
<th>Control and trained combined</th>
<th>Young and old combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
</tr>
<tr>
<td>Pre-synaptic remodeling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axon withdrawal (%)</td>
<td>39.9 ± 4.6</td>
<td>40.9 ± 5.2</td>
</tr>
<tr>
<td>Axon sprouts (%) (n)</td>
<td>2.3 ± 0.8</td>
<td>9.0 ± 2.3 (^a)</td>
</tr>
<tr>
<td>Terminal Schwann cell processes (%)</td>
<td>14.3 ± 2.5</td>
<td>19.9 ± 3.7</td>
</tr>
<tr>
<td>Size &amp; pre/post relationship</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve terminal volume (um3)</td>
<td>76.6 ± 8.9</td>
<td>74.4 ± 9.5</td>
</tr>
<tr>
<td>Motor endplate volume (um3)</td>
<td>224.4 ± 8.5</td>
<td>247.9 ± 8.4</td>
</tr>
<tr>
<td>Overlap (%)</td>
<td>33.9 ± 4.3</td>
<td>34.3 ± 4.7</td>
</tr>
<tr>
<td>Endplate stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median dispersion volume (%)</td>
<td>39.4 ± 1.3</td>
<td>37.2 ± 1.0</td>
</tr>
<tr>
<td>Median \textit{en face} dispersion (%) (^1)</td>
<td>10.9 ± 0.5</td>
<td>13.5 ± 0.7 (^i)</td>
</tr>
<tr>
<td>Fragmented endplates (%) (^1)</td>
<td>11.6 ± 1.4</td>
<td>28.7 ± 2.5 (^a)</td>
</tr>
<tr>
<td>Agrin overlap (%)</td>
<td>76.9 ± 5.4</td>
<td>72.2 ± 6.2</td>
</tr>
</tbody>
</table>
\(^a\) Significant difference \((p \leq 0.05)\) from young group
\(^i\) Significant interaction \((p \leq 0.05)\) between age and training
\(^1\) Log transformed to achieve homoskedasticity; values shown untransformed
\(^a\) Non-parametric testing completed due to heteroskedasticity

Values are means +/- standard error; \(n = 40\) (20 per group)
Table 4. Main effects of age and training on NMJs in the medial TA

<table>
<thead>
<tr>
<th>Pre-synaptic remodeling</th>
<th>Control and trained combined</th>
<th>Young and old combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon withdrawal (%) n</td>
<td>18.5 ± 3.6</td>
<td>15.6 ± 3.8</td>
</tr>
<tr>
<td>Axon sprouts (%)</td>
<td>2.7 ± 0.8</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>Terminal Schwann cell processes (%)</td>
<td>8.6 ± 1.5</td>
<td>17.8 ± 2.8 a</td>
</tr>
<tr>
<td>Size &amp; pre/post relationship</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nerve terminal volume (μm³)</td>
<td>88.8 ± 10.0</td>
<td>127.5 ± 9.1 a</td>
</tr>
<tr>
<td>Motor endplate volume (μm³)</td>
<td>101.9 ± 4.0</td>
<td>116.3 ± 4.4 a</td>
</tr>
<tr>
<td>Overlap (%)</td>
<td>64.8 ± 4.9</td>
<td>71.1 ± 4.2</td>
</tr>
<tr>
<td>Endplate stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median dispersion volume (%) n</td>
<td>34.7 ± 1.4</td>
<td>44.4 ± 2.0 a</td>
</tr>
<tr>
<td>Median en face dispersion (%)</td>
<td>8.5 ± 0.5</td>
<td>12.0 ± 0.7 a</td>
</tr>
<tr>
<td>Fragmented endplates (%) 1</td>
<td>19.9 ± 2.1</td>
<td>34.2 ± 2.6 a</td>
</tr>
<tr>
<td>Agrin overlap (%) n</td>
<td>95.7 ± 1.3</td>
<td>93.8 ± 2.9</td>
</tr>
</tbody>
</table>

a Significant difference (p ≤ 0.05) from young group

1 log transformed to achieve homoskedasticity; values shown untransformed

n Non-parametric testing completed due to heteroskedasticity

Values are means +/- standard error; n = 40 (20 per group)
Effects of age

Pre-synaptic remodeling was greater in the old group than in the young adult group, as reflected by axon sprout and Schwann cell process percentage (Figure 3.3). In the lateral TA, the old group had a significantly higher percentage of axon sprouts than the young adult by approximately 7% (W = 122, p=0.02). In the medial TA, the old group had a doubling of the percentage of terminal Schwann cell processes found in young adult (F$_{[1,36]}$=8.94, p=0.005). No significant effects of age were found in the percentage of axon withdrawal.

There was a significant main effect of age on the volume of both nerve terminals and motor endplates in the medial TA; the old group had a larger nerve terminal volume (F$_{[1,36]}$=7.90, p=0.008) and motor endplate volume (F$_{[1,36]}$=5.77, p=0.02) than the young adult group. A similar trend was seen in the motor endplate volume in the lateral TA (F$_{[1,36]}$=3.91, p=0.06). Aging had no significant effect on synaptic overlap in either the lateral or medial TA.
Figure 3.10. Micrographs of motor endplates (red) and nerve terminals (yellow) from rats in (A) the young trained group and (B) the old control group, demonstrating the larger volume in both the motor endplate and nerve terminal in the old group compared with the young group, as well as the increased *en face* dispersion and fragmentation in the old control group (scale bars = 5 μm).

The old group showed greater motor endplate instability than the young adult group, as reflected in measures of dispersion and fragmented motor endplates. The old group had a higher *en face* dispersion percentage than the young adult group in the medial TA ($F_{[1,36]}=17.23$, $p=0.0002$). In the lateral TA, there was a significant interaction between age and training ($F_{[1,36]}=4.64$, $p=0.04$) demonstrating that *en face* dispersion differences between young adult and old were only seen in the control group. This is discussed further below under training effects. The old group in the medial TA also had a higher dispersion volume than the young adult group ($W = 73$, $p=0.0004$). A main effect of age was found in the percentage of fragmented motor endplates; in the lateral TA, the old group had 17% more fragmented motor endplates than young adult ($F_{[1,36]}=10.81$, $p=0.002$), and in the medial TA the old group had 14% more fragmented endplates than...
young adult ($F_{1,36}=10.80$, $p=0.002$). No significant differences were found between age groups in the percentage of agrin overlap, although the old control group had the lowest percentage of agrin overlap in both the lateral and medial TA.

**Effects of training**

Although no significant effects of training or interactions between age and training were found in pre-synaptic remodeling measures, examination of effect sizes revealed a trend in the percentage of axon withdrawal in both the lateral and medial TA (Figure 3.11). An opposite relationship between age groups was seen within the control and trained groups; the young adult control group had a lower percentage of axon withdrawal than old controls, while the percentage of axon withdrawal in the young adult trained group was greater than in old trained group.

No significant effects of training or interactions between age and training were found in nerve terminal volume, motor endplate volume, or the parentage of synaptic overlap.
Figure 3.11: Effect sizes with confidence intervals of comparisons between young adult and old within each experimental group (open circle = control group, filled circle = trained group), demonstrating axon withdrawal was less in the young adult control group than in the old control group, but greater in the young adult trained group than in the old trained group. This observation was not statistically significant in group comparisons.
As noted previously, there was a significant interaction in the lateral TA between age and training that showed the effect of age on *en face* dispersion was limited to the control group (Figure 3.12). The old control group had a higher *en face* dispersion than the young adult control group (p=0.0007), but there was no difference in *en face* dispersion between old and young adult within the trained group (p=0.5). There was no effect of training on median *en face* dispersion in the medial TA, or on dispersion volume or agrin overlap in either the medial or lateral TA.

**Figure 3.12.** Maximum z-projection images demonstrating the difference in *en face* dispersion between motor endplates from (A) an old rat in the trained group and (B) an old rat in the control group. In image B, note the greater unstained black space between the red-stained acetylcholine receptor clusters compared with image A. Also note the increased fragmentation of the motor endplate from the old control rat (scale bars = 5µm).
Figure 3.13: Significant interaction of age and training on en face dispersion in the lateral TA; within the control group, the old group had a higher en face dispersion than the young adult group. There was no difference between age groups within the trained group. Data are shown as mean and standard error.
Differences in variance

Differences in variance between the old trained and old control groups accounted for 6 of the 7 variables that did not pass Levene's test of equal variance (Figure 3.14). In the lateral TA, the old control group had higher variability than the old trained group in median en face dispersion (p=0.02) and percentage of fragmented endplates (p=0.03). Similarly in the medial TA, the old trained group had higher variability in percentage of axon withdrawal (p=0.023), median dispersion volume (p=0.002), and percentage of agrin overlap (p=0.008). An opposite effect of training was seen in the percentage of fragmented endplates in the medial TA, with the old trained group having higher variability than the old control group (p=0.0002). The homoskedasticity in the remaining variable, percentage of axon sprouts in the lateral TA, was due to an effect of age, with higher variability in the old group than the young adult group (trained and control groups combined) (p=0.0003).
Figure 3.14: Plot of residuals showing training significantly decreases variability in the old group (range of residuals in old trained is less than old control) in all plotted variables except fragmentation in the medial TA, which shows the opposite effect. (L-TA = lateral TA; M-TA = medial TA).
3.3.3 Relationship between USVs and NMJs

Pearson product-moment correlation coefficient calculations revealed significant relationships between acoustic measures (simple and complex USVs combined) and combined measures of NMJ morphology from both lateral and medial TA (Figures 3.15 – 3.17). The effects of age were also demonstrated by the data distributions found in Figures 3.15 – 3.17.

Most notably, peak amplitude was negatively correlated with both median *en face* dispersion \( (p=0.0004, r=-0.53, 95\% \text{ CI}: -0.72 \text{ to } -0.26) \) and percentage of fragmented endplates \( (p=0.005, r=-0.43, 95\% \text{ CI}: -0.66 \text{ to } -0.14) \). Conversely, USV duration was positively correlated with percentage of fragmented endplates \( (p=0.002, r=0.47, 95\% \text{ CI}: 0.19 \text{ to } 0.68) \).
Figure 3.15: USV peak amplitude was inversely related to NMJ en face dispersion. Amplitude is reported in dBFS, with a range from -96 minimum to 0 dB maximum.
Figure 3.16: USV peak amplitude was inversely related to percentage of fragmented endplates in NMJs. Amplitude is reported in dBFS, with a range from -96 minimum to 0 dB maximum.
Figure 3.17: USV duration was positively related to the percentage of fragmented endplates in NMJs.
3.4 Discussion

The hypothesis of this research was that vocal training would reverse age-related changes in USVs and laryngeal NMJs of senescent rats. This hypothesis was tested by comparing rat USV acoustics, NMJ morphology in the lateral and medial TA, and the relationship between these measures. Results showed: 1) age-related differences existed in both USV acoustics and NMJ morphology, 2) some of these differences were mitigated by vocal training, and 3) significant relationships existed between measures of USV acoustics and measures of NMJ motor endplate stability. Accordingly, the results of this study supported the hypothesis.

3.4.1 Effects of age

The effect of age on USVs was evidenced by smaller amplitude, higher frequency, and longer duration of USVs in the old group than in the young adult group. In TA NMJs, aging was associated with increased pre-synaptic remodeling, larger volumes of the pre- and post-synaptic components, and increased motor endplate instability. Significant relationships were found between these age-related changes; USV amplitude was negatively related to both \textit{en face} dispersion and endplate fragmentation, and USV duration was positively related to endplate fragmentation. Therefore, aging was associated with both acoustic changes in USVs and reduced motor endplate stability and impacted the relationship between these measures (Figures 3.15-3.17).
These age-related differences in USVs are consistent with the one other study on acoustic changes in senescent rat vocalizations (Basken et al., 2012). In that study, the old group of F344/BN rats had a lower average intensity of both simple and FM USVs, lower frequency of simple USVs, and increased duration of step USVs compared with a young adult group (in the current study, FM and step vocalizations were analyzed together as complex vocalizations). The longer duration of complex USVs in the old group found in both studies may have been an attempt to compensate for the overall reduced USV intensity, as hypothesized by Basken et al. (2012). It may also indicate a decrease in the fine motor control needed to begin and end USVs. Loss of fine motor control may result from an increase in the size of the motor unit seen with aging (Larsson & Ansved, 1995). Indeed, acoustic changes in vocalizations from old rats have been associated with a decrease in the number of primary motoneurons in the nucleus ambiguus, suggesting motor unit remodeling with age (Basken et al., 2012). This interpretation is supported by EMG findings from the human TA that show longer motor unit durations in older participants (Takeda et al., 2000). The signs of pre-synaptic remodeling of the NMJ seen in the old group in the current study are likely another sign of this age-related motor unit remodeling. In fact, age-related changes at the NMJ may be a primary cause of motor unit remodeling (Santo Neto, & Marques, 2008).

Because different types of USVs have different social and communicative intent (Knutson, Burgdorf, & Panksepp, 2002), these acoustic changes likely have functional
and social implications. It is possible to examine the social implications of USVs through preferential and self-selection playback experiments, as has been done to explore the connection between 50-kHz USVs and appetitive behaviors and reward (Burgdorf et al., 2008). Future research based on the results of this current study will examine the functional and social implications of age-related changes in USV acoustics.

The age-related changes in the NMJ are similar to an earlier study of aging NMJs in the rat TA that found age-related increases in the number of terminal Schwann cell processes and qualitative signs of receptor cluster degradation (Connor et al., 2002). These signs, described as receptor clusters (motor endplates) that appeared "diffuse, distributed over a large area," are similar to the results of the current study that found increased percentage of fragmented motor endplates and increased motor endplate dispersion in the old group. Therefore, aging was associated with changes in NMJ morphology in the rat TA. Although NMJ physiology and function were not directly examined in this study, the implications of these age-related changes in endplate stability are an increased potential for synaptic failure due to the disruption of the pre/post-synaptic relationship (Balice-Gordon, 1997a; N. Robbins, 1992; Smith, 1979; Smith, 1984). Reliable synaptic transmission at the NMJ depends on precise structural arrangements of pre- and post-synaptic elements (Slater, 2003). Synaptic failure at the NMJ may manifest functionally as muscle weakness and fatigue, which is likely why changes in NMJ morphology precede clinical signs of sarcopenia (Deschenes et al.,
Accordingly, findings from the present study are consistent with previous research and with well-known sensorimotor sequelae of aging.

### 3.4.2 Effects of training

The effect of training on USVs was evident by the higher post-training vocalization rate in the trained group than the control group. Additionally, the trained group had no age-related difference in USV amplitude, while the baseline age effect of lower USV amplitude in old persisted in the control group. Vocal training also mitigated an effect of aging on the NMJ by reducing en face dispersion in the old trained group. Therefore, vocal training changed vocal behavior by increasing vocalization rate and reduced age-related effects on USV acoustics and neuromuscular mechanisms in the TA, a muscle used in USV production.

Another effect of training was decreased variability in measures of motor endplate stability in the old trained group relative to the old control group. This suggests the effect of training may have been stronger on certain individual animals than on group means. Studying the same NMJ in vivo over time in a single animal may provide a more accurate picture of the effect of training. Repeated in vivo imaging has been used with more superficial muscles (Balice-Gordon, 1997b), but the challenges of difficulty with accessibility and small size have not yet been overcome in the larynx.
Interestingly, the effect of training on dispersion was only seen in the *en face* dispersion measure, not in measure of volume dispersion. Differences between two- and three-dimensional measures of the NMJ were also demonstrated in Study B. It is possible that the measurements are capturing different phenomena. Another possibility is that measuring dispersion in two dimensions has more physiological relevance than measuring it in three dimensions. The post-synaptic motor endplate lies relatively flat along the muscle fiber. Measuring *en face* dispersion captures the increased area taken up by the motor endplate as it expands along the muscle fiber, whereas adding the third dimension may actually obscure this measure. The *en face* dispersion measure does, however, take advantage of the three dimensionality of the confocal microscopy technique by first rotating the NMJ to a standardized *en face* orientation. Therefore, *en face* dispersion is the recommended measurement technique to quantify the age-related expansion of the motor endplate.

### 3.4.3 Agrin

This is the first study to investigate the effects of age and exercise on agrin in any skeletal muscle. If the amount of agrin and/or localization of agrin with the motor endplate were to be decreased in aging muscles, measures of endplate stability and aggregation, such as endplate fragmentation and dispersion, would be expected to increase (as they did in this study). However, results did not show a significant difference in agrin/motor endplate overlap. It is possible the particular agrin measurement in this
study was not sensitive enough to detect changes. It is also possible that other factors underlie age-related motor endplate fragmentation and dispersion. Agrin is only one of four primary factors in the muscle fiber basal lamina that contribute to NMJ maintenance (Fox et al., 2008). In addition to agrin, the basal lamina contains β2-laminin, collagen α3/6 (IV), and nidogen-2. Therefore, future research should examine these other factors to explore other mediators of aging synaptic stability and plasticity.

3.4.4 Comparison between lateral and medial TA

There were similar age-related effects in the lateral and medial TA, although there were different manifestations and magnitudes of these effects. Training impacted the lateral and medial TA differently as evidenced by the reduction of en face dispersion in the trained group in the lateral TA, but not the medial TA. This suggests the lateral TA is more involved with USV production than the medial TA.

Differences in the effects of age and training between the lateral and medial may also be due to differences in form and function of the two muscle portions. Division between lateral and medial TA is a common feature in mammals (Rhee & Hoh, 2008). The lateral TA is known as the external or muscularis portion, and is critical for laryngeal closure, while the medial TA is known as the vocalis portion, and is active in regulating tension in the vocal fold during phonation in humans (Hirano, 1974). The demands placed on the medial TA during phonation are likely responsible for its complex
innervation (Sanders, Wu, Mu, Li, & Biller, 1993). Further division of the medial vocalis has been suggested as an explanation for pitch control (Sanders, Rai, Han, & Biller, 1998). Functional differences of the medial and lateral TA in rat USVs, however, are unknown. Important morphological have been identified between the two divisions. The lateral portion is larger than the medial portion and homogeneous in its muscle fiber type distribution, while the medial TA is more heterogeneous (Rhee, Lucas, & Hoh, 2004). This difference in muscle size and muscle fiber type may account for the differences observed in the size of the motor endplates, as both fiber size and type influence NMJ morphology (Prakash et al., 1996; Slater, 2008b). There also is a difference in the distribution of motor endplates between the lateral and medial TA; in this study endplates of the lateral TA were found to be organized into an endplate band, similar to other skeletal muscles, while the medial TA endplate distribution was more diffuse (Figure 3.9). These observations underscore the importance of considering the two portions separately when examining the effects of age and/or an intervention.

3.4.5 Factors influencing USV acoustics

The relationships between USV acoustics and NMJ morphology implicate age and training-related changes in laryngeal neuromuscular biology contribute to changes in USV acoustics. However, the NMJ is only part of a cluster of central and peripheral changes that are likely affected by exercise and aging. Age-related changes both within the larynx and throughout the body likely impact USVs. For example, changes in
laryngeal tissue composition and biomechanics in humans have been implicated as causes for acoustical changes in the senescent voice (Hammond et al., 2000; M. Hirano et al., 1989); the impact of age on laryngeal biomechanics in the rat is unknown. Additionally, USVs are produced with an egressive airflow and, therefore, age-related changes in breathing and pulmonary function likely contribute to changes in USVs. Studies of human speech breathing have shown older adults use different respiratory mechanisms when producing speech, likely due to changes in the strength of respiratory musculature, compliance of the chest and lungs, and lung volume (Hoit & Hixon, 1987; Huber & Spruill, 2008). Changes in rat pulmonary function with aging has been identified (Johanson & Pierce, 1973), although subglottal pressure during USV production has only been studied in young rats (Riede, 2011). Lastly, the paradigm used to elicit and train USVs relied on the male rats' interest in a female. Therefore, age-related decline in mating behavior may have affected age-related differences in USVs (McGinnis & Yu, 1995). Despite the multitude of factors possibly contributing to changes of USVs with age and exercise, the results from this study provide the first evidence for a relationship between USVs and NMJs.

One of the primary variables in this study that was affected by age and exercise was USV amplitude. When measuring changes in amplitude, variations in distance from the sound source to the microphone will result in variations of amplitude. In this study, rats were allowed to roam freely in their home cage while vocalizations were recorded.
One reason for the difference in amplitude between ages could be that young adult rats are generally more active than old, and, therefore, may have vocalized closer to the microphone, thus increasing their average amplitude. If this were the case, the variation in the amplitude of young adult rats should have been higher than in old. There was no difference, however, between young adult and old groups in amplitude variability. Additionally, variability of individual rats was accounted for by taking an average measurement of many vocalizations for each rat. Therefore, the observed age-related differences in USV amplitude are likely due to a true difference between age groups and not variations in mouth to microphone distance.

In addition to an effect of vocal training, there may have been an effect of the control condition, suggested by the lower vocalization rate in the old control group compared with the young adult control group. Both the trained and control groups were kept socially isolated (singly housed) throughout the 8 weeks to improve response to vocal training (Panksepp & Burgdorf, 2000). Also, because rats produce USVs in social contexts, they likely had little reason to vocalize when socially isolated. In recordings over a 5 hour period, male rats housed in pairs were found to vocalize approximately 10 times more when left in the same cage during the recording period than when recorded in isolation (Figure 3.18). Although limiting vocalization outside of the training sessions allowed for more control over total vocal use, it may have created a social isolation/vocal disuse effect in the control group. This may also be the reason post-training amplitude
was smaller than at baseline in both the training and control groups. Future studies will be necessary to confirm this possible effect and to characterize the impact of social isolation on the laryngeal muscles.

3.4.6 Conclusion

This is the first study to show vocal training can reduce the effects of advanced age on both USVs and NMJ morphology in the rat TA. It also demonstrated how using a rat model of behavioral vocal training can help elucidate the neuromuscular changes with age in the voice and how voice use and training may function to assist in mitigating these changes.
Figure 3.18. Comparison of the average number of vocalizations per hour during a 5-hour recording session showing the increased number of vocalizations in doubly-housed rats when recorded together, compared with the same rats (rat A and rat B) when recorded separately. The singly-housed rat (recorded alone) had the lowest number of vocalizations.
Chapter 4: Overall Conclusion

The overall goal of this research was to investigate the effects of age and exercise on NMJs in muscles of swallowing and voice. Two different models of exercise in the cranial muscles, bilateral NMES of the hypoglossal nerves innervating muscles of the tongue and behavioral vocalization training to activate and exercise muscles of the larynx, were shown to effect age-related changes in NMJs of the GG in the tongue and the TA in the larynx. Additionally, the effects of age and exercise differed both between muscles, as well as within the lateral and medial portions of the TA.

4.1 Comparison of aging and exercise in tongue and larynx

Statistically significant signs of pre-synaptic remodeling were not observed in any age group in the GG, either with or without NMES. It was hypothesized at the completion of Study A that this result may have been due to the novel three-dimensional measurement technique employed in that study (see discussion in Study A). In contrast to the traditional two-dimensional imaging used previously in other muscles (Son & Thompson, 1995b), our approach allowed three-dimensional rotation of the NMJ to examine and rule out phenomena that may have been erroneously judged as Schwann cell processes or axon sprouts when viewed in only two-dimensions. However, three-dimensional imaging of the TA in Study B did reveal presynaptic remodeling as a function of age in the form of increased percentages of Schwann cell processes and axon
sprouts in the old group. These different results in GG and TA muscles suggest that three-versus two-dimensional imaging was likely not a factor contributing to characterization of pre-synaptic morphology in the GG, but was a reflection of inter-muscle differences. A lack of signs of age-related NMJ pre-synaptic remodeling in the GG was consistent with a previous study (Hodges et al., 2004). One possible reason for this difference between muscles may be that aging and exercise-related changes in the NMJ have been shown to differ between functionally and structurally diverse muscles (Alshuaib & Fahim, 1990; Deschenes et al., 1994; Prakash & Sieck, 1998).

The GG and TA have shared as well as distinct functions in the upper airway. Both are active during swallowing (Van Daele et al. 2005), with the GG activating early in the swallow to generate oral pressure (Palmer et al., 2008), and the TA becoming active slightly later to close the glottis and protect the airway (Perlman, Palmer, McCulloch, & Van Daele, 1999). As a relatively large tongue protruser, the GG also has a role in airway patency for unobstructed respiration (Sauerland, 1975). The airway patency and swallowing functions of the GG muscle would require rapid contraction as well as endurance in the completion of these tasks. The TA, on the other hand, is a small intrinsic laryngeal muscle that is active during laryngeal adduction to modulate airflow during expiratory tasks such as phonation, coughing, and laryngeal braking during exhalation of large tidal volumes (Hiroto, Hirano, Toyozumi, & Shin, 1967; Kuna, Insalaco, & Woodson, 1988), and requires very rapid contraction speeds for voicing and
airway protection. These different functional demands are reflected in the differences in each muscle’s fiber type composition.

The GG and TA of the rat differ in myosin heavy chain (MHC) isoform composition. The rat GG is comprised of type IIa, IIx, and IIb MHC isoforms and a small percentage of type I MHC (Schaser et al., 2011). Additionally, there are differences in MHC distribution along the anteroposterior axis of the GG (Schaser et al., 2011). The findings of Schaser et al. (2011) were reported after the GG samples for Study A had been obtained, and the precise location within the GG of these samples was unknown. The rat TA consists exclusively of type IIx, IIb, and IIeo (extra-ocular) MHC as well as hybrid combinations of these isoforms (Inagi et al., 1999; Rhee et al., 2004; T. Suzuki et al., 2002). Type IIeo is also called IIL in the rat larynx (Merati, 1996). NMJs on type I and type IIa muscle fibers have been shown to be smaller in area and less affected by age than NMJs on type IIx and IIb muscle fibers (Deschenes et al., 1994; Ogata & Yamasaki, 1985; Prakash et al., 1996), possibly explaining the fewer age effects seen in the GG versus the TA.

The primary effect of NMES in the old group in the GG was a decrease in the motor endplate volume. In the TA, training did not affect motor endplate volume but decreased endplate fragmentation. The differences between these two training effects may be due to the different modalities used to model exercise (NMES vs. behavioral
training). In studies of contractile properties of the GG, NMES was shown to increase muscle force, while behavioral training increased muscle endurance (Connor, Russell, Kletzien, Schaser, & Leversong, March 3-5, 2011). Differences in muscle recruitment during nerve stimulation and voluntary contraction may contribute to this; nerve stimulation is designed to elicit a maximal muscle contraction by recruiting all muscle fibers, whereas voluntary muscle contraction rarely reaches maximum muscle contractile capability and varies among individual contractions (Lowe & Alway, 2002). Also, different training intensities of the same type of intervention in young animals have different effects on the NMJ in the soleus muscle, with high intensity training inducing greater hypertrophy of the NMJ than low intensity training (Deschenes et al., 1993). The effect of high intensity training (NMES) had the opposite effect (decrease in volume) in old rats in the GG (Study A), confirming the effects of exercise are not only muscle-dependent, but also age-dependent (Deschenes et al., 2011).

The volume of both the pre- and post-synaptic components among the lateral TA, medial TA and GG muscles was different, regardless of age or exercise (Figures 4.1 and 4.2). Differences in staining techniques are likely partially responsible for differences in nerve terminal volumes between the GG and TA. In Study A, the pre-synaptic component of GG NMJs included neurofilaments, terminal Schwann cells, and synaptic vesicles of the nerve terminal. In Study B, a new technique became available that allowed those same three elements to be imaged with separate fluorescent labels. Because of this
improved discrimination ability, the pre-synaptic component of the TA in Study B included only the nerve terminal membrane. Thus, pre-synaptic volumes cannot be directly compared between studies, but are plotted for the reader’s consideration (Figure 4.2). In contrast, high affinity staining of the acetylcholine receptor clusters in the motor endplate using α-bungarotoxin was held constant between the two studies, suggesting TA motor endplates have a smaller volume and are less variable in size than GG motor endplates (Figure 4.1).

Differences in motor endplate volumes may be due to differences in muscle fiber type between the GG and TA. As outlined above, in limb muscles, NMJs on type I and IIa muscle fibers tend to be smaller and less complex than NMJs on type IIx and IIb muscle fibers (Deschenes et al., 1994; Ogata & Yamasaki, 1985; Prakash et al., 1996). Based on these prior findings from the limbs, an increased proportion of type I and type IIa muscle fibers in the GG would predict smaller NMJs relative to those in the TA that is largely composed of type IIx and IIb muscle fibers. However, the findings from the present studies suggest the opposite (Figure 4.1). While NMJ size can be positively related to muscle fiber size (Slater, 2008b), age-related changes can be exhibited independent of changes in muscle fiber diameter (Rosenheimer & Smith, 1985). Collectively, these results underscore the importance of studying the tongue and laryngeal muscles directly to understand the effects of aging and exercise on the NMJ on swallowing and voice and not rely solely on studies in the limb musculature.
Figure 4.1. Comparison of motor endplate volume between muscles. Note the y-axis is log-transformed to facilitate easier visual comparison of outliers.
Figure 4.2. Comparison of nerve terminal volume between muscles. Note the y-axis is log-transformed to facilitate easier visual comparison of outliers.
4.2 Physiological and functional consequences of alterations in NMJ morphology

The changes in NMJ morphology found in this research may have physiological and functional consequences related to swallowing and voice. Reliable synaptic transmission at the NMJ depends on precise spatial arrangement of the synapse and is necessary for consistent and dependable muscle contraction (Slater, 2003). As was demonstrated in this and other research, age-related changes in NMJ morphology disrupt the synaptic structure, including expansion of both pre- and post-synaptic components and fragmentation and dispersion of the motor endplate; therefore, aging likely affects reliability of synaptic transmission at the NMJ (Balice-Gordon, 1997a; N. Robbins, 1992; Smith, 1979; Smith, 1984). Evidence of this has been seen in physiologic studies of the aging rat tongue, where decreased tetanic tension and increased contraction and recovery times have been reported in old muscles (Nagai et al., 2008; Ota et al., 2005). Physiological study of the aging TA has also shown decreased muscle contraction capability and a higher likelihood for synaptic failure in old rats (McMullen & Andrade, 2009). However, there is potential for NMES to provide treatment for these deficits in muscle fiber contraction. A recent study using NMES investigated the effects of treatment on age-related tongue muscle contractile properties, showing that NMES reduced muscle fatigue and increased twitch and tetanic tension in young adult and old rats (Connor et al., in press). Additionally, a study of chronic NMES of the rat TA muscle reported several neuromuscular adaptations in the TA in response to NMES, including decreased mean muscle fiber area and increased NMJ density, although no muscle contractile properties were reported (McMullen, 2011). These two NMES studies, encompassing both the tongue and larynx, in combination with the results of
Studies A and B, allow us to speculate, in part, on biological processes underlying the changes in muscle contractile properties evidenced following NMES. Altogether, the changes in NMJ morphology and muscle physiology associated with exercise, either voluntary or induced through NMES, may improve synaptic transmission and, therefore, increase muscle strength and improve muscle function as evidenced by the relationship between decreased motor endplate stability and USV amplitude.

Recent studies have reported age-related deficits in functional sensorimotor behaviors for swallowing and vocalization using a rat model. For instance, evidence from videofluorographic study of aging rats reported deficits in swallowing function with aging (Russell et al., in press). The results of this dissertation showing the effect of age on USV acoustics confirm evidence that vocal function in rats is affected by age (Basken et al., 2012). While some of the deficits in swallowing and voice found in these studies are similar or analogous to age-related changes in human swallowing and voice, it was not the goal of this dissertation to compare rat vocalization and swallow to human behaviors. As noted in a highly relevant article concerning the use of animal models in neuroscience, it is more important in translational research to establish how a particular functional deficit might be expressed within a particular animal than to attempt a direct correspondence to the human condition (Cenci, Whishaw, & Schallert, 2002). Therefore, establishing the effects of age and exercise on swallowing and vocalization in the rat will strengthen the use of the rat model for studying connections between behavior and underlying neuromuscular mechanisms in the tongue and laryngeal sensorimotor systems.
4.2 Conclusion

Interventions in speech-language pathology are behavioral in nature, although electrical stimulation therapy for the treatment of dysphagia has been recently added to the armamentarium of the speech-language pathologist in some settings. While the goals of treatment are functional, they seek to restore function by affecting underlying biological mechanisms. Studying the effects of behavioral interventions on underlying neuromuscular mechanisms in the cranial muscles is a unique challenge because of the small size and inaccessibility of these muscles. The results of this research have demonstrated how two models of exercise, NMES of the tongue and behavioral vocal training, affect neuromuscular plasticity in the senescent muscles of swallowing and vocalization. Understanding the neuromuscular mechanisms underlying age and exercise in the cranial muscles is critical in the development and testing of potential interventions for age-related dysphagia and dysphonia. This translational research has provided meaningful findings that can be used for the development of hypotheses to test in human participants and pave the way for evidence-based interventions.
References


