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

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Abstract

INTRODUCTION—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.

METHODS—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 versus untreated controls.

RESULTS—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.

CONCLUSIONS—In the NMJ, aging and NMES are associated with changes in the pre- and post-synaptic relationship.

Keywords

Neuromuscular junction; NMJ; electrical stimulation; aging; tongue

Introduction

Dysphagia has been identified as a common and chronic problem in the aging population.¹ Age-related changes in the tongue likely contribute to muscle weakness and fatigue, and, consequently, dysphagia.^{2–6} However, age-related changes in the tongue muscles and the response of these muscles to different treatments have been understudied.^{7–9}

Age-related morphologic changes at the neuromuscular junction are similar to changes found in the denervation-reinnervation process.^{10–12} Aging changes the size, complexity, and relationship of pre- and post-synaptic neuromuscular junction (NMJ) morphology.^{11, 13–16} The physiologic sequelae of age-related morphologic changes include reduced synaptic transmission efficiency and increased fatigue.^{10, 14, 17–19} 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.^{20–22} In physical therapy, lower limb rehabilitation NMES in combination with voluntary exercise has been shown to be more effective than voluntary exercise alone.²³ 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.²⁴ Although initial studies have shown some benefit from this approach,²⁵ thorough evidence of its efficacy is lacking.²⁶

There is a paucity of research on how NMES affects underlying neuromuscular mechanisms. While it has been shown that exercise changes the size and complexity of both pre- and post-synaptic structures of the NMJ,^{27–30} little is known about the effects of electrical stimulation on NMJ morphology.^{31–33}

Most of the research on the relationships among aging, exercise and NMJ morphology has been performed in animal hindlimb muscles^{17, 34, 35} or the diaphragm,³⁶ while little work has focused on the cranial muscles.^{11, 14, 15} 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.^{37, 38} Additionally, there are age-related changes in muscle contraction times and maximum force generation between limb and tongue muscles.³⁹ Direct study of cranial muscles is critical to a complete understanding of age-related muscles 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.^{40, 41} Maintaining three-dimensionality may provide more precise measurements of NMJs. However, the consistency of nerve terminal and motor endplate measurements made in two dimensions versus 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.

Materials and Methods

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the NIH 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 (IACUC) of the University of Wisconsin-Madison School of Medicine and Public Health.

Animal subjects and experimental design overview

A total of 50 male Fischer 344/Brown Norway rats are reported from three age groups; 9-month-old (young adult, $n=18$), 24-month-old (middle aged, $n=13$), and 32-month-old (old, $n=19$). This strain of rats has a 33-month median lifespan.⁴² 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).

Electrode implantation and stimulation protocol

Animals in the stimulation group were surgically implanted with a prefabricated electrode assembly⁴³ consisting of a skin plug for connecting to an external stimulator, 6 lead wires, and 2 stimulation electrode cuffs placed around the hypoglossal nerve on each side. To simulate a clinical exercise paradigm used to strengthen the tongue muscles⁴⁴ 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 sec stimulation train at 40 Hz followed by 1.0 sec of rest, with a 2 minute 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 μ A). 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° C.

Immunohistochemistry

At a later time, whole muscles were fixed in 4% formaldehyde for 1 hour at room temperature. Longitudinal, 50 μ m thick sections were cut from the midsection of each muscle and mounted directly onto a slide coded such that the microscopist (AMJ) 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.^{10, 11, 15} To label acetylcholine receptors within the motor endplate, samples were incubated in 20 μ g/mL of tetramethylrhodamine-conjugated α -bungarotoxin (Invitrogen: Molecular Probes, Eugene, OR) in PBS for 30 minutes 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, CA), 1:100 anti-synaptic vesicle (SV2, mouse IgG1, Developmental Studies Hybridoma Bank, Iowa City, IA), and 1:250 anti-S-100 labeling both myelinating and non-myelinating terminal Schwann cells (S-100, rabbit IgG, Dako, Carpinteria, CA). The next day, samples were incubated for 4 hours in 1:100 CY5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) and 1:100 Alexa Fluor 488-conjugated donkey anti-rabbit (Invitrogen: Molecular Probes, Eugene, WA).

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, NY). 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 μm spacing and 1 μm optical section depth were collected for each of the three colors.

Image processing

Image stacks were processed using ImageJ.⁴⁵ After examining a three-dimensional reconstruction of each NMJ, an assistant (AS) blind 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 below) were automatically calculated using a custom ImageJ macro (AMJ). First, an automatic thresholding algorithm (Auto threshold v. 1.8, 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. Motor endplate concentration ratio was calculated by dividing the motor endplate volume by the volume of the convex hull.²⁸

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 to 13. The median number of slices per image stack was 49, with a range 21 to 89.

Statistical analysis

Analysis of Variance (ANOVA) was used to test age effects, stimulation treatment effects, and age by 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 pair-wise comparisons were made between groups using Fisher's protected least significant difference tests (LSD).

Linear regression was used to examine the relationship between nerve terminal volume and motor endplate volume for each animal. ANOVA was then used to test for differences in the

root mean square error and the slope of the regression as a function of age or stimulation treatment. Linear regression was also used to examine the relationships between volume and area measurements and between two- and three-dimensional measurements of motor endplate concentration ratio.

All analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC). The critical value for obtaining statistical significance was set at $\alpha=.05$ level.

Results

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.

Quantitative morphologic measurements

Motor endplate volume, nerve terminal volume, and concentration ratio by age and stimulation treatment group are summarized in Figure 1. A significant age by 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 below. No significant main or interaction effects were found for nerve terminal volume or endplate concentration ratio.

Post hoc LSDs 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), 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 ($p = 0.01$) of motor endplate volume than did old control animals (Figure 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).

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 3. Results of the ANOVA revealed a significant interaction effect between age and stimulation treatment for the slope of the regression lines ($F_{[2,44]} = 3.66$, $p = 0.03$) (Figure 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 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. Post hoc LSDs showed the slopes of both the young adult and middle aged control groups were significantly different than the slope of the old control group ($p < .001$ and $p = 0.003$, respectively).

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. Relative to control animals, 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.

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 to 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 to 0.98). Data from these regressions are summarized in Figure 5.

ANOVA results for area measurements were different than those for volume measurements reported previously in this paper. 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 to 0.95). Although results of the ANOVAs performed with the two- and three-dimensional measurements of endplate concentration ratio yielded different p-values, neither analysis showed statistically significant main or interaction effects for aging or stimulation.

Discussion

This study shows 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. Additionally, 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.

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 lab in which a non-significant decrease in receptor area was found between young adult (9 months) and old (36 months) animals (middle-aged animals were included in that study).¹⁵ Rosenheimer and Smith¹⁹ 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). Non-linear 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 to young rats.⁴⁶ 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 indicated 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 age-related decline in neuromuscular function in the genioglossus muscle of the tongue.

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).⁴⁷ Increased postsynaptic folding has been shown to amplify the effect of acetylcholine on muscle activation by doubling the safety factor for neuromuscular transmission.⁴⁸ Additionally, it has been suggested that the degree of postsynaptic folding is inversely related to the size of the motor endplate.^{49, 50} 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 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 Pette 2001),^{51, 52} as well as increase muscle fiber cross-sectional area.⁵³

It is possible that changes in the muscle fibers may have accounted for the morphological 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.²⁸ showed that changes in NMJ morphology as a result of resistance exercise training were independent of changes in muscle fiber size. Rosenheimer and Smith¹⁹ 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).³⁶ We did not visualize different muscle fiber types in this study, but other studies in our lab 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).

Clinical implications

Current clinical practice using NMES for the treatment of dysphagia uses surface electrodes, not direct nerve stimulation.²⁴ However, because swallowing involves a complex temporal interaction of multiple cranial muscles, treatment of dysphagia with surface electrodes may provide limited benefit. 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.⁵⁴ 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.²³

Depending on the underlying cause of the swallowing deficit, therefore, direct nerve stimulation 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 suggest it is a safe and effective treatment for sleep apnea.⁵⁵ Our study used direct nerve stimulation to ensure that the tongue muscles were being stimulated. The morphological 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 in the sleep apnea literature and the results of this study, further investigation of direct nerve stimulation for the treatment of dysphagia is recommended.

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 studies⁴⁰ 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 $170 \mu\text{m}^2$, but with volumes of 243, 314, 353, 436 and $670 \mu\text{m}^3$. 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, while three-dimensional measurements are consistent despite changes in orientation. Figure 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 degrees. The area of the motor endplate in the original orientation is $75 \mu\text{m}^2$, while the rotated endplate has an area of $133 \mu\text{m}^2$. 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 three-dimensional measurements more accurately and consistently capture the size of NMJ morphology.

Contrary to previous studies of NMJ morphology both in our lab^{11, 15} and by others,^{10, 11} 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.^{11, 13, 17} 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.¹⁵ Other pre-synaptic parameters have been observed to change with aging, such as increased nerve terminal branching,¹³ and these parameters may provide further insight into age-related changes of NMJs in the tongue.

In summary, 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 postsynaptic 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.

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Abbreviations

ANOVA	Analysis of Variance
LSD	Fisher's protected Least Significant Difference tests
NMJ	Neuromuscular Junction
NMES	Neuromuscular Electrical Stimulation

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Figure 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.

Two-dimensional projections of neuromuscular junctions from control animals 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 animals demonstrating the decrease in motor endplate size associated with stimulation treatment in the old group. (scale bar = 10 μ m)

Figure 3.

Plots of the regression lines of the relationship between nerve terminal and endplate volume for each animal. Each box presents data from one animal. 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 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 animals, a positive mean slope was significantly different from the Old Control animals ($p = .03$).

Figure 5. Box and whisker plots of the R^2 values from the regressions between two- and three-dimensional measures.

Figure 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 degrees on the y-axis. The stained area in (A) is 43% smaller than the stained area in (B). (scale bar = 10 μm)