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ORIGINAL RESEARCH

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Utilizing novel recurrent laryngeal motor nerve conduction studies to characterize the aging larynx: A pilot study

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Abstract

Objectives: Age-related changes to the larynx are associated with dysphonia and contribute to reduced quality of life. This study utilizes recurrent laryngeal motor nerve conduction studies (rlMNCS) to determine if neurophysiologic changes occur in the aging larynx using an aging rat model.

Study Design: Animal study.

Methods: In vivo rlMNCS were performed in 10 young hemi-larynges (3–4 months) and 10 aged hemi-larynges (18–19 months) rats (Fischer 344 × Brown Norway F344BN). Recording electrodes were placed into the thyroarytenoid (TA) muscle through direct laryngoscopy. Recurrent laryngeal nerves (RLNs) were directly stimulated with bipolar electrodes. Compound motor action potentials (CMAPs) were obtained. RLN cross-sections were stained with toluidine blue. Axon count, myelination, and g-ratio were quantified utilizing AxonDeepSeg analysis software.

Results: rlMNCS were successfully obtained in all animals. Mean CMAP amplitude and negative durations in young rats were 3.58 ± 2.20 mV and 0.93 ± 0.14 mS (mean dif: 0.17; 95% CI: −2.21 to 2.54), respectively, and 3.74 ± 2.81 mV and 0.98 ± 0.11 mS (mean dif: 0.050; 95% CI: −0.07 to 0.17). No significant differences in onset latency or negative area were observed. Mean axon count in young rats (176 ± 35) was comparable to that in old rats (173 ± 31). Myelin thickness and g-ratio did not differ between groups.

Conclusions: There were no statistically significant differences in RLN conduction or axon histology between young and aged rats in this pilot study. This work provides a basis for future, adequately powered studies, and may lead to a tractable animal model to study the aging larynx.

Level of Evidence: 5.

KEYWORDS

age-related vocal atrophy, aging voice, compound motor action potential, motor nerve conduction studies, Neurolaryngology, recurrent laryngeal nerve

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1 | INTRODUCTION

Aging affects the structure and function of the larynx.[1–5] Age-related changes include vocal fold bowing, persistent glottal gap, reduced mucosal wave, and increased compensatory muscle involvement.[4,6–9] This constellation of features is referred to as age-related vocal atrophy (ARVA).[10] ARVA is associated with meaningful dysphonia and has significant impact on the quality of life of elderly patients.[11,12] As of now, the pathophysiologic mechanisms of ARVA are still being described and no one treatment has been proven effective in preventing or improving dysphonia in patients with ARVA.[13,14] The neurophysiologic correlates of ARVA in particular have yet to be characterized.

The presence and mechanisms of age-related changes in other neuromuscular systems have been well described using a combination of histology, electromyography (EMG), and motor nerve conduction studies (MNCS), especially in the hands and limbs.[15,16] At the level of the muscle, a combination of EMG and muscle testing has shown decreases in muscle tone, force generation, contractility, and coordination in aged individuals. In correlation with this, histologic methods have demonstrated redistribution of Types I and II muscle fibers, smaller and decreased motor fibers, and reduced size and number of neuromuscular junctions (NMJs).[17–19] At the level of the nerve, there are reductions in axon count, decreased myelination, and reductions in motor units.[20] Histologic studies in the larynx have demonstrated altered mucosal properties as well as reductions in size and abundance of NMJs in the thyroarytenoid (TA) and posterior cricoarytenoid muscles; however, neurophysiologic testing of the larynx has been limited to EMG recordings.[21,22] Laryngeal EMG may have utility in the management of vocal ford paralysis and paresis; however, its utility in ARVA has not been described.[23–25] Although laryngeal EMG provides useful information about the neuromuscular control of voice, it relies on volitional control and does not precisely isolate the end neuromuscular deficit in the larynx.

MNCS may provide valuable insight into the nerve-to-muscle connectivity in the larynx. MNCS have been shown to detect changes that occur in aging and in neuromuscular diseases and are frequently utilized in diagnosis and monitoring of neuromuscular diseases such as carpal tunnel syndrome.[17,26–34] The authors recently developed a paradigm for conducting MNCS in the rat larynx, termed recurrent laryngeal motor nerve conduction studies (rIMNCS) in which an electrode is placed into the TA muscle under direct laryngoscopy and the recurrent laryngeal nerve (RLN) is stimulated to obtain compound motor action potentials (CMAPs), motor unit number estimations (MUNEs), and nerve conduction velocities (NCVs). The amplitude, duration, latency, and area of the CMAP provide valuable information regarding the integrity and coordination of nerve–muscle conduction. These metrics change significantly in the setting of injury, disease, and aging and serve as both useful diagnostic measures as well as outcome measures for monitoring progression and treatment response.

The primary aim of this study was to investigate the neuromuscular changes to the 18 month aged larynx using rIMNCS and histological measures of axons characteristics in an aged rat model. We hypothesized that the aged rat larynx would demonstrate diminished CMAP amplitude, prolonged CMAP duration, and decreased axon myelination.

2 | MATERIALS AND METHODS

This animal study was conducted in accordance with the Public Health Service policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Ace (7 U.S.C. et seq.). The Institutional Animal Care and Use Committee at the University of Washington School of Medicine approved the animal use protocol.

A total of 20 hemi-larynges from Fischer F344 × Brown Norway rats (National Institute on Aging rodent colony) were used in this study; 10 from 4-month old rats and 10 from 18-month old rats. The animals were housed and maintained in a limited-access facility, with 12-h light/dark cycles. The facility staff assessed the animal well-being and offered rat chow and water ad libitum.

2.1 | Recurrent laryngeal motor nerve conduction studies

Prior to each experiment, the rat was weighed and anesthetized by intraperitoneal injection of a ketamine (50 mg/kg) and xylazine (5 mg/kg) cocktail. The anesthetized rat was secured to an angled intubation stand (Kent Scientific, Torrington, CT) in supine position. Direct laryngoscopy was achieved using a nasal speculum secured to an adjustable ring stand. An operative microscope (Leica M80 stereomicroscope with on Axis NVI illumination and 175-mm objective focal length) was used to visualize the larynx. A 27-gauge monopolar needle electrode (Ambu Neuroline, Columbia, MD) was placed transorally in the TA laryngeal muscle that comprises the vocal fold. The reference electrode was placed in the posterior pharyngeal wall and the ground electrode was placed into the tail. An incision was made in the midline from the larynx and inferiorly toward the sternal notch. Strap musculature was divided to visualize bilateral RLNs. A 2-mm bipolar stimulating electrode (Cadwell, Kennewick, WA) was secured and positioned directly on the RLN 5 mm below the level of the cricoid cartilage. The nerve was stimulated using single monophasic pulses (1–2 Hz) starting at an intensity of 0.1 mA (pulse duration 0.1 mS). The stimulation intensity was increased by 0.1 mA increments until the negative (upward) amplitude reached a plateau. This defined the CMAP. An additional stimulus at an intensity 30% above the intensity at which the CMAP was obtained was given to confirm that additional intensity did not change the CMAP amplitude. If the CMAP had an initial predominant downward deflection, the active electrode was repositioned. Stimulation intensity, latency of onset, peak negative amplitude, negative duration, and negative area were recorded. Supramaximal stimulation was repeated three times and trace data were averaged for each animal. The stimulating electrode was then moved proximally to a distance of 15 mm from the cricoid and the

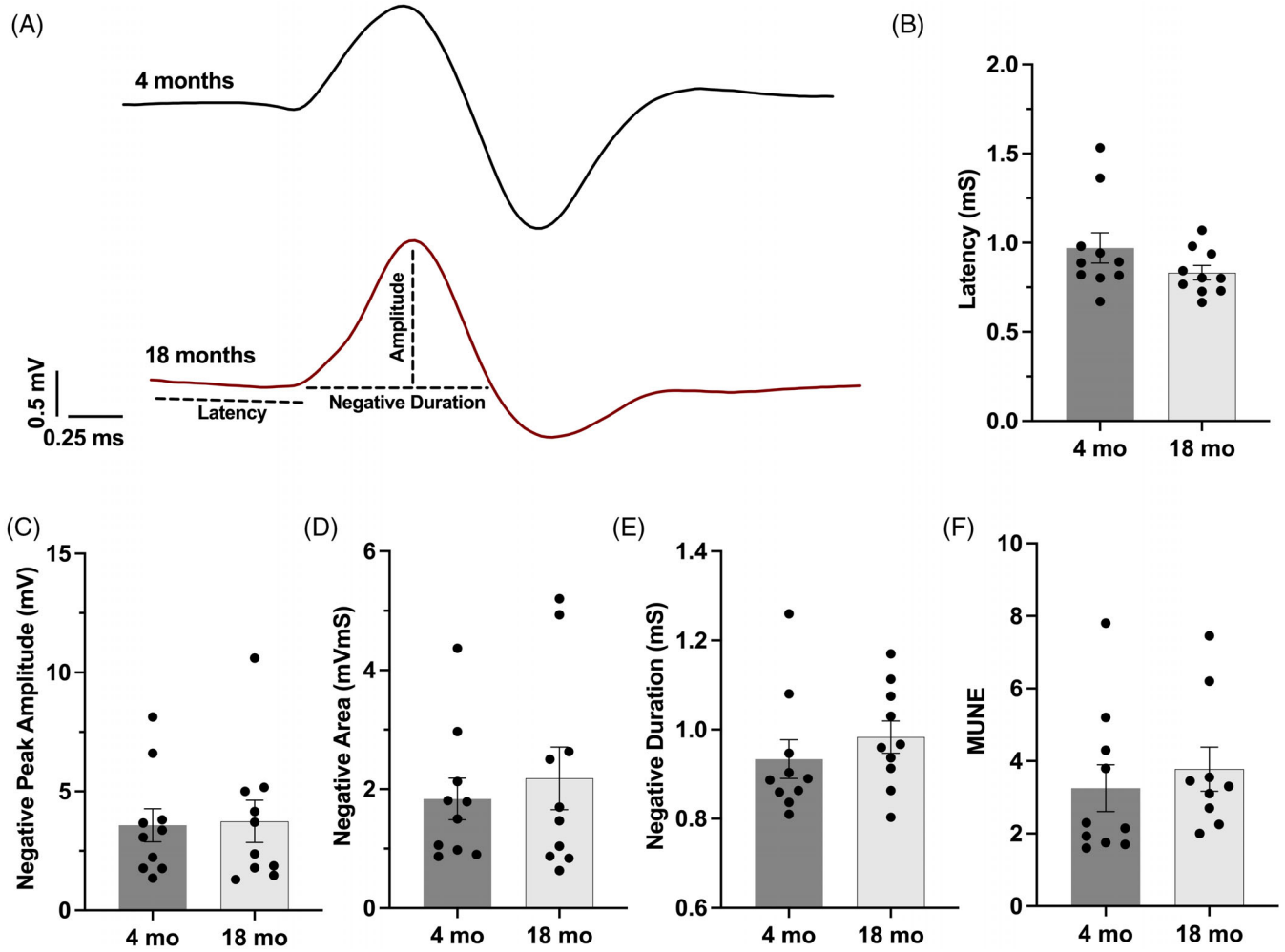

FIGURE 1 Comparison of CMAP properties at 4- and 18-months. Mean and SEM shown. (A) Example CMAP traces, (B) Latency: time to CMAP onset after stimulation, (C) Negative peak amplitude: amplitude from baseline to maximum negative deflection, (D) Negative Area: area under negative curve, (E) Negative Duration: time from initial negative deflection to subsequent crossing of baseline, (F) MUNE: estimated number of motor units. CMAP, compound motor action potential; MUNE, motor unit number estimate.

experiments were repeated. The NCV was obtained by dividing the difference in latencies by the distance between stimulation sites measured with a caliper.

MUNE was performed using the incremental stimulation method described by McComas et al.[35–37] Briefly, once the intensity necessary to achieve the CMAP was determined, the RLN was serially stimulated using submaximal stimulation intensity. Stimulation intensity was increased from 0.0 by increments of 0.03 mA until the motor unit potential amplitude increased. This was recorded as the single motor unit potential (SMUP) amplitude. Serial stimulation with increasing intensity was conducted until 10 separate SMUPs were calculated. These were averaged to determine the mean SMUP. The CMAP amplitude (negative amplitude) was divided by the SMUP (negative amplitude) to determine the MUNE. Calculations were made automatically by the Sierra Summit software (Cadwell, Kennewick, WA). This was repeated two times for each nerve and reported data are the average.

2.2 | Tissue processing and histology

Following the motor evoked study, rats were euthanized by intraperitoneal injection of pentobarbital (200 mg/kg). Bilateral RLNs were harvested and fixed in 10% buffered formalin solution for 48 h before being transferred to 1× phosphate buffer solution (PBS); 2 mm nerve sections were washed in fresh PBS before post-fixation in osmium tetroxide. The osmium was rinsed with distilled water before samples were dehydrated through a graded series of acetone, infiltrated with epon-araldite resin, and embedded. Blocks were trimmed and semi-thin (7 mm) sections were placed onto glass slides and counter stained with toluidine blue. Images from three separate axial cuts were acquired with a Zeiss brightfield microscope (Zeiss, Oberkochen, Germany) and a 40× air lens. Images were post processed in ImageJ™ and axon measurements were made using an automated open-source software called AxonDeepSeg (ADS) as previously described[38] Briefly, ADS will automatically segment images into axon and myelin layers

TABLE 1 CMAP comparisons between 4- and 18-month-old rats.

	4 months ($n = 10$)	18 months ($n = 10$)	Mean difference; 95% CI	p Value
Intensity (mA)	0.72 ± 0.21	1.49 ± 0.55	−0.76; −1.18 to −0.35	.01
Latency (mS)	0.97 ± 0.27	0.83 ± 0.13	−0.14; −0.34 to 0.059	.16
Negative amplitude (mV)	3.58 ± 2.20	3.74 ± 2.81	0.17; −2.21 to 2.54	.89
Negative duration (mS)	0.93 ± 0.14	0.98 ± 0.11	0.050; −0.069 to 0.17	.39
Negative area (mVmS)	1.84 ± 1.11	2.18 ± 1.67	0.35; −0.98 to 1.68	.59
MUNE	3.25 ± 2.03	3.78 ± 1.83	0.52; −1.36 to 2.41	.56

Abbreviations: CI, confidence interval; CMAP, compound motor action potentials; MUNE, Motor Unit Number Estimate.

based on density and create masks of each. Minimal manual correction was performed to assure accuracy of masks. ADS will then morphometrics including axon number, axon and myelin diameter and area, and the ratio of myelination to axon diameter, known as the g-ratio.

3 | RESULTS

A representative CMAP trace for both 4- and 18-month rats is shown in Figure 1A. Stimulation intensity, latency, negative peak amplitude, negative duration, and negative area were acquired from three trials and averaged. The average CMAP and MUNE data and comparisons between 4- and 18-month-old rats are displayed in Table 1. Scatter plots demonstrating the means and distributions are displayed in Figure 1B–F.

Stimulation intensity varied across individuals ranging from 0.50 to 2.30 mA with a mean of 1.49 mA in 18-month and 0.72 mA in 4-month rats. Stimulation intensity varied across individual animals and may be related to the precise location of the stimulating electrode. Despite varied stimulation intensity, the CMAP was confirmed with a supramaximal stimulus (+30%) to assure that that all motor axons were stimulated. Supramaximal stimulus did not change the CMAP amplitude in any case. Onset latency was similar in young and aged rats (0.97 compared to 0.83, mean dif: −0.14; 95% CI: −0.34 to 0.059). The mean CMAP amplitude at 4 months was 3.58 mA ± 2.20 and 3.74 ± 2.81 in 18-month-old rats and did not significantly differ (mean dif: 0.17; 95% CI: −2.21 to 2.54). Negative duration of the CMAP was 0.93 ± 0.14 and 0.98 ± 0.11 in 4- and 18-month-old rats, respectively, and also did not significantly differ (mean dif: 0.050; 95% CI: −0.07 to 0.17). Negative area was 1.84 ± 1.11 and 2.18 ± 1.67 in 4- and 18-month-old rats, respectively, and did not differ between groups (mean dif: 0.35; 95% CI: −0.98 to 1.68). The average MUNE was around 3 for both groups (3.25 ± 2.03 and 3.78 ± 1.83, respectively).

Seven RLNs were processed in both 4- and 18-month-old rats. A representative image and AxonDeepSeg mask are shown in Figure 2A,B. Axon morphometric data are reported in Table 2 and represent average data from three serial transverse slices from each animal. Mean and distribution are demonstrated in scatter plots in Figure 2C,D.

The average axon count was 175 ± 35 in 4-month-old and 173 ± 31 in 18-month-old rats and did not differ significantly between groups (mean dif: −2.7; 95% CI: −41 to 36). Other metrics also did not differ including axon diameter (mean dif: 0.020; 95% CI: −0.0010 to 0.040), myelin thickness (mean dif: 0.0052; 95% CI: −0.0038 to 0.014), and g-ratio (mean dif: 0.011; 95% CI: −0.035 to 0.056).

4 | DISCUSSION

ARVA is a prevalent condition affecting the larynx of older individuals leading to dysphonia and reduced quality of life. The precise neurophysiologic contributions to ARVA are still unclear, partially due to lacking quantitative measures of RLN conduction properties. This pilot study utilized a newly developed technique for rIMNCS in an animal model to compare neuromuscular conduction properties in aged rats (18-months) and non-aged (4-months) rats in effort to delineate the neurophysiologic contributions to ARVA. Specifically, it was hypothesized that CMAP amplitude would be decreased, whereas onset latency and negative duration would be increased in aged rats compared to non-aged rats. It was also suspected that axon number and the degree of myelination (represented by g-ratio) may be reduced in coordination with these suspected nerve conduction changes. In this pilot study, CMAP measures did not significantly differ between groups (Figure 1; Table 1), nor did axon morphometric measurements (Figure 2; Table 2).

These results contrast with prior work establishing age-related changes in the musculature of the larynx. McMullen and Andrade, identified numerous changes to the TA muscle in aged Fischer 344 × Brown Norway F1 hybrid rats, the same as those used in this study. Specifically, they observed a doubling in muscle fiber area from 6 to 18 months of age. By 30 months, TA muscles generated lower twitch and tetanic forces. Cellular changes were also observed including increased intracellular glycogen-positive fibers, mitochondrial clusters, and ragged red fibers.[39] Subsequent work by McMullen and Andrade demonstrated a significant decrease in NMJ end-plate density and size by 30 months.[21] Denervation likely has a prominent role in causing these functional, cellular, and molecular changes. In fact, McMullen et al. demonstrated that stimulation of the right RLN for two 1-h sessions for either 1 or 2 weeks in young rats, resulted in a significant decrease in mean muscle fiber area and an increase in NMJ

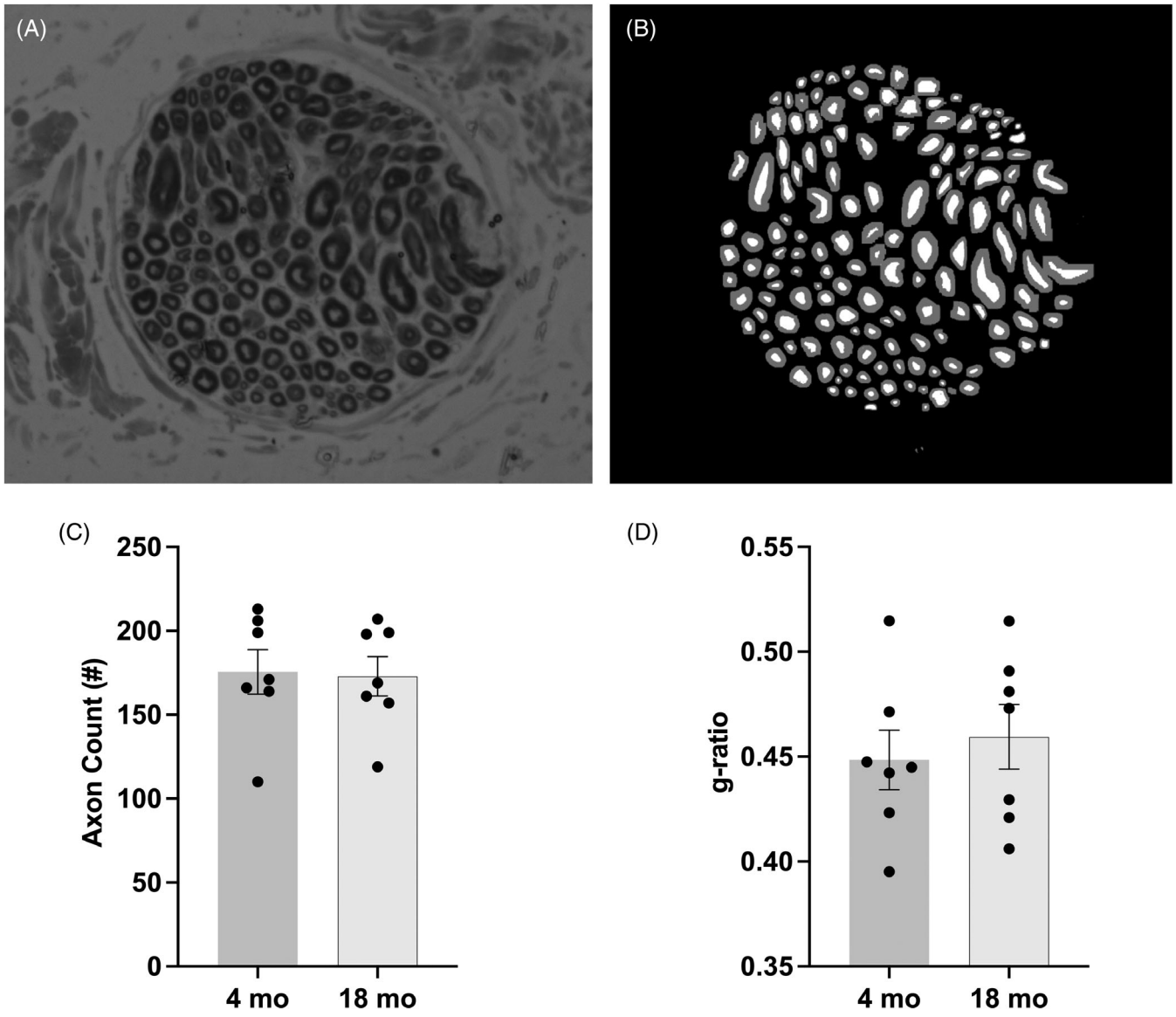

FIGURE 2 Comparison of RLN axon and myelination characteristics. (A) 40× brightfield image of RLN cross-section. (B) Representative binary mask used for axon/mylein quantification. (C) Total axon count, (D) ratio of axon to mylein thickness (g-ratio), (E) Mean myelin thickness. Mean and SEM are shown.

TABLE 2 Axon morphometric comparisons between 4- and 18-month-old rats.

	4 months ($n = 7$)	18 months ($n = 7$)	Mean difference; 95% CI	p Value
Axon count (#)	175 ± 35	172 ± 31	−2.7; −41 to 36	.88
g-ratio	0.97 ± 0.27	0.83 ± 0.13	0.011; −0.035 to 0.056	.61
Axon diameter (μm)	0.11 ± 0.016	0.13 ± 0.019	0.020; −0.0010 to 0.040	.061
Myelin thickness (μm)	0.060 ± 0.0050	0.065 ± 0.0096	0.0052; −0.0038 to 0.014	.23

Abbreviation: CI, confidence interval.

density, mitochondrial content, and glycogen-positive fibers compared to the control side.[21]

Age-related changes in nerve conduction properties have also been demonstrated through NCS performed in the limbs. Wu et al. compared anterior tibialis NCS in rats aged 1, 3, 6, 12, 18, and 24 months and observed decreased CMAP amplitudes and increased onset latency at later time points. Additionally, smaller CMAP amplitude was associated with reductions in Types I and IIa muscle fiber types.[17] In humans, Kurokawa et al. compared median and ulnar NCS in young (15–34 years), intermediate (35–64 years),

and old (65–85 years) humans and found reduced CMAP amplitude and area in older individuals.[28] Reduced NCV in both sensory and motor nerves has been observed in older individuals.[40]

Despite the absent statistical difference between the groups, these pilot data provide useful insights for future investigation. First, nerve conduction changes in the rat larynx may not become prominent until after 18 months. Indeed, NCS in the rat anterior tibialis nerve and human median and ulnar nerves demonstrated a nonlinear relationship between reduced CMAP amplitude and age with amplitude reductions becoming more significant after 18 months and 65 years, respectively.[17,27,41] Furthermore, much of aging research utilizes rats >18-months old.[17,21,39] Thus, future studies comparing 4- and 30-month-old rats may reveal age-related changes to RLN conduction. Second, any neurophysiologic changes that occur by 18 months are likely small and may have not been detected in this pilot study given the small sample size. Onset latency trended toward being longer at 18 months signifying slower nerve conduction, but this finding did not reach statistical significance (Table 1; 95% CI: −0.34 to 0.059; $p = .16$). However, these pilot data provide an effect size that can be used to design an adequately powered future study to characterize the neurophysiologic changes that occur in the larynx by 18 months.

There is uncertainty as to how the electrophysiologic measurements translate to laryngeal function. A necessary future step will be linking electrophysiologic changes to anatomic and functional changes in the larynx. Rat vocalizations have been previously studied and may be a useful measure when performing rIMNCS. Johnson et al.[42] studied the effects of vocal exercise in both young and aged rats.[32] The authors measured the duration of ultrasonic vocalizations (USV) between rats that underwent vocal exercise and a control group. Rats in the control group had shorter USV compared to the vocal exercise group. The USV provided a functional measure that was associated with changes to the NMJ in the larynx. Moving forward, there may be value in using USV or other functional measures to provide context to electrophysiologic changes observed in different experimental conditions. In humans, evaluation morphologic changes to the aging larynx, such as vocal fold bowing, have been correlated with dysphonia and neuromuscular disease.[43] Attempting to make a similar association in rats may be possible with the use of endoscopic laryngeal evaluation and may help link neurophysiologic changes with ARVA. Putting together these pieces that define ARVA could result in a tractable model for developing prevention and treatments.

4.1 | Limitations

The results of this study must be considered in the context of prominent limitations. First, as described above, small sample size limited the power of the comparisons and thus potentially failed to identify statistical differences between groups. Second, with the current technique, limited visibility of the TA made it difficult to place the recording electrode in precisely the same location in each experiment. Future use of small caliber telescopes may help improve placement. Surface temperature may have varied based on the depth of anesthesia and other factors, which could have influenced nerve conduction properties.

Overall, this is a delicate method with some key sources of variability that may have washed out any small differences that exist between 4- and 18-month-old rats. Going forward, further refinement of this technique with strict temperature regulation, endoscopic-guided electrode placement, and repeated measures may provide the added precision necessary to reveal the expected age-related changes to laryngeal neuromuscular conduction.

5 | CONCLUSION

In this pilot study, rIMNCS and RLN axon histology revealed no significant statistical differences in nerve conduction or axon properties when comparing 4- and 18-month-old rats. Neuromuscular conduction changes occurring by 18 months may be small and thus require a larger study to elucidate. Furthermore, future studies comparing older rats (24–30 months) to young rats may reveal a greater magnitude of difference in rIMNCS and can help identify the precise timing of neurophysiologic changes in the aging larynx.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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