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Neuromuscular junction transmission failure is a late phenotype in aging mice

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Abstract

Neurodegeneration has increasingly been considered an important factor in the pathogenesis of sarcopenia or age-related loss of muscle mass and strength. Experiments were designed to investigate the fidelity of neuromuscular junction (NMJ) transmission across the lifespan in hindlimb muscles of male and female C57BL/6J mice (at 12, 20, 24, 27, and 29 months of age). Single-fiber electromyography recordings demonstrated abrupt onset of NMJ transmission failure at 27 months of age. Failed NMJ transmission was a later onset phenotype as compared with other assessments of motor unit numbers, muscle contractility, and frailty which showed alterations at 20 months of age. Ex vivo NMJ recordings demonstrated no reduction of endplate current amplitude in support of reduced muscle fiber excitability as the cause of failed NMJ transmission in aged mice. Improved understanding of age-related neurodegeneration will likely have important implications in designing novel therapeutic interventions specific for different stages of sarcopenia. Our findings suggest reduced muscle excitability may be a potential therapeutic target for improvement of physical function in older adults.

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Authors' contributions

DC, CI, PB, MMR, and WDA contributed to conception and design of the study; DC, CI, PB, XW, MMR, and WDA contributed to acquisition and analysis of data; DC, MMR, WDA contributed to drafting a significant portion of the manuscript or figures; WDA and MMR had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosure

The authors have no conflicts of interest to report.

Keywords

Aging; Neuromuscular junction; Transmission; Motor unit; Sarcopenia; Frailty

1. Introduction

Sarcopenia, defined as abnormal loss of muscle mass and function during aging, is a major contributor to loss of physical function in older adults. As early as the third decade of life, humans can experience progressive loss of muscle strength and mass, and after the sixth decade, muscle loss is more rapid, accelerating even faster after the age of 80 years (Goodpaster et al., 2006; von Haehling et al., 2010). While to some extent, losses of muscle mass and strength are inevitable during aging, inactivity, comorbid medical conditions, and other incompletely understood factors contribute to an increased risk (von Haehling et al., 2010). The initial usage of the term sarcopenia was solely focused on muscle mass, but it has become increasingly clear that muscle strength is a more critical contributor to physical function (Clark and Manini, 2008). There has long been interest in the degenerative consequences of aging on the neuromuscular system, and in recent years, age-related neurodegeneration has increasingly been considered in the context of sarcopenia (Clark and Manini, 2008; Delbono, 2003; Kwan, 2013; Pannérec et al., 2016).

As the final output of the nervous system to skeletal muscle, the effects of aging on the neuromuscular junction (NMJ) have garnered significant interest (Taetzsch and Valdez, 2018). Morphological analyses of aged mice have consistently shown postsynaptic features of fragmentation, loss of junctional folds, and denervation starting at the age of about 18–21 months of age (Chai et al., 2011; Cheng et al., 2013; Taetzsch and Valdez, 2018). Morphological investigations in older adult humans have demonstrated similar but mixed findings (Jones et al., 2017; Oda, 1984; Wokke et al., 1990). Two prior studies demonstrated features of NMJ fragmentation and fewer junctional folds in older people with a mean age of 50–60 years (Oda, 1984; Wokke et al., 1990). In contrast, a more recent study demonstrated remarkable stability in lower limb muscles of ~60-year-old human patients (Jones et al., 2017). One potential caveat of the more recent study by Jones et al. is that the controls for these experiments were obtained during limb amputation, which were for the most part performed for complications related to diabetes and peripheral vascular disease (Jones et al., 2017). Both diabetes and peripheral vascular disease are associated with peripheral nerve pathologies such as generalized neuropathies and compression mononeuropathies (Juster-Switlyk and Smith, 2016; McDermott, 2015). Therefore, the consequences of aging on human NMJ morphology remain unresolved.

Several studies have performed electrophysiological analyses of NMJ transmission in aged rodent models. Surprisingly, NMJ recordings from different muscles (extensor digitorum, extensor digitorum longus, soleus, gluteus maximus, and diaphragm) from very old C57BL/6J mice (26–35 months) have shown features of age-dependent enhancement of endplate potential (EPP) or endplate current (EPC) when compared to younger animals (Banker et al., 1983; Fahim, 1993; Willadt et al., 2016). Furthermore, Willadt et al investigated NMJ transmission in the diaphragm muscle in aged C57BL/6J mice (26–28

months) and showed the lack of association between NMJ fragmentation and endplate response in the diaphragm of very old C57BL/6J mice (Willadt et al., 2016). Therefore, the consequences of aging on NMJ morphological alterations have not been shown to have functional implications. One potential limitation of NMJ preparations assessing EPP or EPC is that the sufficiency of endplate response to trigger action potential is not directly tested. Single-fiber electromyography (SFEMG) is a technique that can be used to assess the fidelity of NMJ transmission to trigger action potential responses (Stalberg and Trontelj, 1997). SFEMG is a clinically relevant technique and is the most sensitive test for clinical diagnosis of NMJ transmission defects (Stalberg and Trontelj, 1997). SFEMG is performed using an extracellular electrode to measure single-fiber action potentials (SFAPs) after voluntary contraction or axonal stimulation (Stalberg and Trontelj, 1997). Jitter is an SFEMG parameter that assesses the variability of NMJ transmission latency between consecutive action potentials and is increased with pathology at the NMJ (Stalberg and Trontelj, 1997). Blocking is an SFEMG parameter that characterizes failure of action potential generation during SFEMG measurements. There have been no prior clinical SFEMG studies performed specifically in the context of aging or sarcopenia, but there is some evidence that NMJ transmission declines in healthy older adults on the basis of increased jitter cutpoint reference values for older adults (>60 years of age) in a large clinical reference database (Bromberg and Scott, 1994). Similarly, there is some evidence for increased jitter in aged C57BL/6J mice (20–24 months), but blocking was not specifically analyzed in these studies (Chung et al., 2017, 2018).

In this study, we were interested in investigating SFEMG jitter and blocking across the lifespan of C57BL/6J mice. A key unanswered question regarding age-related alterations of the NMJ is whether NMJ changes are related to a process directly involving the NMJ or are these alterations related to degeneration occurring elsewhere such as the muscle fiber or the motor neuron. Loss of spinal motor neurons and ventral root axons and alterations of the NMJ morphology were demonstrated decades ago in older adults (Arizono et al., 1984; Kawamura et al., 1977; Mittal and Logmani, 1987; Oda, 1984; Tomlinson and Irving, 1977; Wokke et al., 1990). Similar to anatomical motor neuron counts, a decline in electrophysiological motor unit number estimation (MUNE) have been demonstrated in older adults (Brown, 1972; Campbell et al., 1973; Doherty et al., 1993; McNeil et al., 2005). In mice, there have been mixed findings regarding age-related anatomical losses of motor neurons in the spinal cord (Chai et al., 2011; Maxwell et al., 2018; Valdez et al., 2010). We recently demonstrated that motor unit loss is an early phenotype in aging mice starting at the age of 20 months and that these changes were closely associated with muscle size and muscle contractility (Sheth et al., 2018). Another recent study showed loss of sensory synapses on motor neurons in the spinal cords of 27-month-old male mice compared with spinal cords from 3-month-old mice (Krishnan et al., 2018). The discrepancy in motor neuron cell body losses may be attributable to different timescales of aging between species where humans age for more than 30 years (~60–90 years) as compared with only about 9 months in mice (21–30 months) (Barns et al., 2014; Dutta and Sengupta, 2016). In addition, there are a number of degenerative changes that occur in muscle, and these have been suggested to trigger secondary changes in NMJ morphology (Li et al., 2011). Molecular analyses of aging skeletal muscles have revealed several changes that are associated with

NMJ denervation and protein metabolism in mice (24 months old) as well as in rats (21–27 months old) (Barns et al., 2014; Ibebunjo et al., 2013). Similar age-dependent changes are reported in sciatic nerves from aging mice where accumulation of proteins and electron-dense aggregates are prominent within axons in peripheral nerves suggesting impaired mechanism for axonal transport and protein turnover (Krishnan et al., 2016). Taken together, several age-dependent morphological, molecular, and/or physiological changes are demonstrated at motor neuron, NMJ, and the skeletal muscle levels. However, the inter-relationships between these changes are still very elusive.

On the basis of prior clinical and preclinical findings, the work described here was designed to address the following questions: (1) Do aged mice demonstrate features of failed NMJ transmission (action potential blocking) during SFEMG recordings? (2) When do NMJ transmission defects emerge during the lifespan of C57BL/6J mice? and (3) How does burden of NMJ transmission failure relate to loss of muscle contractility, motor unit numbers, and clinical frailty? In the present study, we hypothesized that NMJ transmission failure would occur concurrently with the onset of loss of muscle contractility and motor unit loss in aged mice. We also hypothesized that mice with increased clinical frailty and loss of muscle contractility would have more prominent findings of NMJ transmission failure and motor unit losses.

2. Methods

2.1. Animals

The animal protocol was approved by and complied with the animal care and ethics guidelines of The Ohio State University Wexner Medical Center. All studies were approved by the Animal Institutional Care and Use Committee of The Ohio State University. A total of 70 C57BL/6J mice (42 males, 28 females) were used for all experiments at 12–13 (referred to as 12 months), 19–21 (referred to as 20 months), 24, 27, and 29 months of age and were procured from the National Institute on Aging mouse colony. Experimental groups were balanced for sex in all experiments except for the SFEMG assessment of frequency-dependent jitter and blocking during which predominantly male mice were used (9 male and 4 females) as well as the ex vivo NMJ recordings during which only male mice (7 males) were studied. Table 1 lists all in vivo and ex vivo measures and their respective gender-specific group sizes. All measurements were performed by evaluators who were blinded to mouse age.

2.2. Anesthesia and animal preparation

During electrophysiological and muscle contractility measurements, mice were anesthetized with inhaled isoflurane using the Kent Scientific SomnoSuite System (induction: 2.5%–3.5%, maintenance: 1%–2%) (Torrington, CT, USA). During electrophysiological and SFEMG recordings, animal body temperature was maintained at 37 °C using a thermostatically controlled heating plate (World Precision Instruments, Sarasota, FL, USA). During the muscle contractility measurements, a warm water bath HTP-1500 heat therapy pump (Adroit Medical Systems, Loudon, TN, USA) was set to maintain temperature of the testing stage at 37 °C. During all procedures performed under anesthesia, lubricating

ointment was applied to the eyes to prevent corneal dryness (Dechra Veterinary Products, Overland Park, KS, USA). All measurements were performed on the right hindlimb which was shaved before the studies. All procedures that required anesthesia lasted for no more than 20 minutes.

2.3. Frailty assessments

To understand the relationship between clinical deterioration, motor unit connectivity, and NMJ transmission failure, frailty scores were obtained. A comprehensive 31-item frailty index based on established clinical signs of deterioration was calculated for each animal as previously described (Whitehead et al., 2014). Clinical examination involved evaluation of the integument (fur loss, coat color, coat condition, and dermatitis), musculoskeletal system, vestibulocochlear/auditory system, ocular and nasal systems, digestive system, urogenital system, respiratory system, signs of discomfort, body weight, and body surface temperature. An infrared thermometer was used to record the body surface temperature from the abdomen (average of 3 readings were used) (Etekcity, Anaheim, CA, USA). A simple qualitative scale of 0–1 was used to rate the severity of each deficit. A score of 0 was given if there was no sign of deficit, a score of 0.5 indicated a mild deficit, and a score of 1 was given for a severe deficit. For frailty assessments, an additional cohort of 6-month-old mice ($n = 17$, 5 males and 12 females) was used to serve as reference values for body weight and body surface temperature measurements as described previously (Whitehead et al., 2014). Deficits in body weight and body surface temperature were scored based on deviation from the reference values in the young adult (6-month old) animals.

2.4. Muscle contractility

In vivo plantar flexion torque measurements were performed using an in vivo muscle contractility apparatus (Model 1300A, Aurora Scientific Inc, Canada) as previously described (Sheth et al., 2018; Wier et al., 2018). Briefly, the right hind paw was taped to the force plate and positioned such that the foot and the tibia were aligned at 90°. The knee joint was securely clamped at the femoral condyles while avoiding the compression of the nearby fibular nerve. Two disposable monopolar electrodes (Natus Neurology Inc, Middleton, WI, USA) were inserted subcutaneously over the tibial nerve for stimulation. Maximum plantar flexion tetanic torque (millinewton-meters [mN-m]) was measured using a train of supramaximal 0.2 ms square wave stimuli delivered at 150 Hz.

2.5. Motor unit number estimation

MUNE was determined in the sciatic nerve-innervated triceps surae muscles of the right hind limb using clinical electrodiagnostic systems (Synergy EMG machine version 9.1, Oxford Instruments, Abingdon, UK; Cadwell Sierra Summit, Kennewick, WA, USA) as previously described (Arnold et al., 2015; Wier et al., 2018). Briefly, 2 insulated 28-gauge monopolar needle electrodes (Teca, Oxford Instruments Medical, NY, USA) were inserted subcutaneously at the proximal hind limb in the region of the sciatic nerve for stimulation. Two fine wire ring electrodes (Alpine Biomed, Skovlunde, Denmark) were used for recording, with the active electrode placed just distal to the knee and the reference electrode placed over the metatarsal region of the foot. One disposable strip ground electrode (CareFusion, Middleton, WI, USA) was placed on the tail. Peak-to-peak compound muscle

action potential responses were obtained after supramaximal stimulation of the right sciatic nerve (constant current intensity of <10 mA with a pulse duration of 0.1 ms, 20 mV sensitivity, 10 Hz- 10 kHz filter). Single motor unit potential amplitude was obtained by determining the average of 10 incremental submaximal incremental responses (50–500 μ V sensitivity, 10 Hz-10 kHz filter). MUNE was calculated using the following formula: MUNE = compound muscle action potential amplitude (peak-to-peak)/average single motor unit potential (peak-to-peak).

2.6. Stimulated single-fiber electromyography

Stimulated SFEMG was performed using a clinical electrodiagnostic system with Viking software (Natus neurology Inc, WI, USA). Two insulated 28-gauge monopolar needle electrodes (Natus Neurology Inc, Middleton, WI, USA) were inserted subcutaneously at the proximal hind limb to stimulate the right sciatic nerve at a rate of 10 Hz (constant current intensity of 0.3–10 mA with a pulse duration of 0.1 ms). The filter was set at 1 kHz–10 kHz, and the gain was adjusted from 200 to 1000 μ V per division to visualize potentials. Sweep speed was set to 500 μ s. A disposable strip ground electrode (CareFusion, Middleton, WI, USA) was placed on the left foot. The SFEMG needle (Natus neurology Inc, WI, USA) was inserted in the right gastrocnemius muscle parallel to the long axis of the muscle fibers to record SFAPs while minimizing muscle trauma. The stimulus was adjusted to isolate SFAPs for analyses. To consider a response as an SFAP, the following criteria were required: baseline-to-negative peak rise time of <500 μ s, minimum amplitude (baseline-to-negative peak) of 200 μ V, and a stable all-or-none response with consistent morphology. Jitter was calculated from 50–100 consecutive discharges using peak detection. To avoid inclusion of muscle fiber action potentials triggered by direct muscle stimulation, the sciatic nerve was stimulated at the proximal hindlimb, away from the gastrocnemius muscle. In addition, because direct muscle stimulation is associated with very low jitter values, potentials with jitter <4 μ s were not included in the analyses (Meekins et al., 2007). On average, H unique SFAPs were obtained per animal for the calculation of mean and median jitter. Importantly, while recording potentials with evident blocking (failure of NMJ transmission and action potential generation), stimulus intensity was increased to confirm that submaximal stimulation was not responsible for action potential failure.

2.7. Assessment of frequency dependency of NMJ transmission in aged mice

A separate cohort of mice aged 21–30 months (21–22 months, n = 3 [all males], 24 months, n = 4 [all males], 27–30 months, n = 6 [4 females, 2 males]) were analyzed to investigate the characteristics of NMJ transmission at different frequencies of stimulation. These experiments were designed to address 2 questions: (1) Whether increased rates of axonal stimulation would elicit action potential blocking at synapses that did not demonstrate blocking at 10 Hz stimulation rates (i.e., healthy junctions). (2) Whether junctions with abnormal transmission at 10 Hz (i.e., impaired junctions) would demonstrate either increased action potential blocking or improvement of blocking at higher frequency of stimulation (i.e., rate-dependent increase in blocking or facilitation of transmission). During these recordings, synapses were identified and recorded at 10 Hz, 20 Hz, and 30 Hz while maintaining consistent SFAP morphology to ensure recording electrode position.

2.8. Ex vivo neuromuscular junction electrophysiology

To further investigate the abrupt onset of increased jitter and SFEMG blocking in 27- and 29-month-old mice compared to 24-month-old mice, isolated ex vivo NMJ preparations were performed on the tibialis anterior muscle in 24-month-old ($n = 3$, all males) versus 29-month-old ($n = 4$, all males) mice as previously described (Wang et al., 2004). Briefly, the tibialis anterior muscle was perfused with Ringer solution containing physiologic Ca^{2+} maintained at $20\text{ }^{\circ}\text{C}$ – $22\text{ }^{\circ}\text{C}$ and equilibrated with 95% O_2 and 5% CO_2 . To allow visualization of the nerve terminals and muscle fibers, the muscles were stained with $10\text{ }\mu\text{M}$ 4-Di-2ASP and imaged with an upright epifluorescence microscope (Leica DMR). Then, muscle fibers were impaled within $100\text{ }\mu\text{m}$ of the synapse. To avoid movement related to contraction, muscle fibers were crushed on each end of the synapse and voltage clamped to -45 mV . Using a 2-electrode voltage clamp, spontaneous miniature endplate currents (MEPCs) amplitudes and frequencies were recorded, and EPCs were recorded after axonal stimulation. A train of 10 axonal stimulations at 50 Hz was performed to assess decrement or facilitation of the EPC. Quantal content for each synapse was calculated by dividing the amplitude of the EPC by the average MEPC amplitude.

2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc La Jolla, CA, USA). Data from MUNE, frailty, and tetanic torque followed a normal Gaussian distribution. Hence, parametric one-way ANOVA together with Holm-Sidak multiple comparisons tests were used for statistical comparisons. Jitter data were not normally distributed, and therefore, nonparametric test, Kruskal Wallis, and Dunn's multiple comparison tests were used. The chi-square test was used to compare groups for the presence or absence of SFEMG blocking. Results with p value ≤ 0.05 were considered statistically significant. Ex vivo NMJ parameters were analyzed using unpaired t -test. Spearman correlation analyses were performed to study the inter-relationships between frailty, motor unit connectivity, jitter, muscle contractility, and triceps surae wet muscle mass.

3. Results

3.1. Increased SFEMG jitter and blocking in very old 27- to 29-month-old mice

NMJ function was assessed in vivo across the lifespan of mice using SFEMG, which is considered as one of the most sensitive technique for this application. In the present study, mean jitter was calculated for each animal from an average of 11 potentials per animal. Fig. 1A and B represent superposition and raster plot of individual SFAPs with normal jitter recorded from mouse gastrocnemius muscle. SFAPs with increased jitter and blocking are shown in Fig. 1C and D. In middle aged 12-month-old animals, mean jitter ranged from $5\text{ }\mu\text{s}$ to $17\text{ }\mu\text{s}$ and 3% of potentials exhibited blocking. No significant differences were observed for mean jitter or blocking in mice through 24 months of age, but at 27 and 29 months of age, both mean jitter and blocking were significantly increased (Fig. 2A and B).

3.2. Frequency-dependent worsening of NMJ transmission failure

We analyzed a separate cohort of old to very old mice with SFEMG for characteristics of NMJ transmission at different rates of axonal stimulation (10–30 Hz). In this second cohort of 21- to 30-month-old mice ($n = 13$), we were able to assess a total of 60 synapses at all 3 stimulation rates of 10, 20, and 30 Hz. Blocking occurred in 11 of 60 synapses at 10 Hz, 14 of 60 at 20 Hz, and 15 of 60 synapses at 30 Hz demonstrating a slight but insignificant increased rate of blocking at higher frequencies (chi-square, $p = 0.6585$). These findings suggest that increased stimulation frequency does not significantly increase the ability to detect junctions with blocking as compared with 10 Hz stimulation. The second question that we aimed to address with this cohort of mice was whether junctions with abnormal transmission at 10 Hz would demonstrate increased action potential blocking at higher frequency of stimulation. We analyzed the frequency of blocking at 10, 20, and 30 Hz from the 11 synapses that demonstrated blocking at 10 Hz to test whether increased frequency of stimulation resulted in higher rates of blocking or improvement of blocking (i.e., rate-dependent blocking versus facilitation, respectively). These investigations demonstrated increased action potential blocking at higher frequencies (Fig. 3A–D).

3.3. Age-dependent decline in muscle contractility, loss of motor unit connectivity, and increased frailty and their associations with NMJ transmission alterations

The cohort of mice aged 12–29 months assessed with SFEMG (Fig. 1) were also analyzed for features of muscle contractility, motor unit number, and clinical frailty to understand the timing of the different degenerative consequences of aging and to explore the interrelationships. In contrast to NMJ failure, which emerged only at 27 months, significant alterations were noted in the aged mice at 20, 24, 27, and 29 months for MUNE, muscle contractility, and frailty index (Fig. 4). The interrelationships between measures of normalized tetanic muscle contractility, MUNE, frailty index, triceps surae wet muscle weight, and SFEMG measures of jitter and blocking were analyzed with Spearman correlation coefficients in 27- and 29-month-old mice (Fig. 5). These correlation analyses demonstrated significant associations between MUNE, frailty, and blocking on SFEMG. Furthermore, wet weight of the triceps surae muscle was tightly associated with MUNE and frailty index.

3.4. Ex vivo neuromuscular junction electrophysiology

To further investigate presynaptic and postsynaptic NMJ transmission alterations, we performed selective NMJ electrophysiological recordings. We compared 2 groups, 24- and 29-month-old mice, to compare mice before and after the time point when we observed the abrupt onset of SFEMG findings of increased jitter and blocking (Fig 1). To avoid alterations that could be attributable to differences in muscle fiber sizes between groups, the voltage clamp technique was used to assess EPC amplitude, MEPC amplitude, MEPC frequency, and quantal content (Wang et al., 2004). These recordings demonstrated increased MEPC amplitude, decreased MEPC frequencies, but interestingly, EPC, which represents the most functionally relevant measure for reliability of NMJ transmission, was unchanged (Fig 6). Furthermore, there was no significant difference in the decrement or facilitation of the EPC between the 1st and 10th response (P1/P10) during a train of 10 stimuli at 50 Hz (24 months

= 1.631, n = 72 synapses versus 29 months = 1.571, n = 90 synapses; $p = 0.270$; larger ratio = greater decrement) (not shown in Fig 6). Axonal action potential propagation failure is another possible pathophysiological explanation, in addition to NMJ transmission failure, for blocking of SFAP responses during SFEMG. This would be expected to manifest as abrupt loss of EPC amplitude during ex vivo NMJ assessment, but this was not observed arguing against axonal action potential propagation failure.

4. Discussion

Defining the degenerative events that contribute to age-related loss of physical function is becoming an increasingly important task as life expectancy increases. In this study, we demonstrated findings of failed NMJ transmission in 27- and 29-month-old C57BL/6J mice, but no significant decline in NMJ transmission was noted between 12 and 24 months. We further investigated NMJ transmission using selective ex vivo recordings in 24-month-old mice versus 29-month-old mice to understand what presynaptic or postsynaptic changes resulted in this failure. Interestingly, EPC amplitude was unaltered, and in fact, was slightly accentuated in the 29-month-old mice. This was similar to prior ex vivo studies that have investigated EPP and EPC in mice at 26–35 months of age (Banker et al., 1983; Fahim, 1993; Willadt et al., 2016). Although these changes in the ex vivo NMJ recordings preparations would be expected to increase NMJ fidelity in aged mice, this enhanced presynaptic transmitter release may be adaptive in response to postsynaptic alterations. Of note, ex vivo NMJ recordings are performed after blockage of muscle fiber action potential generation, thereby preventing direct assessment of the fidelity of action potential generation (Willadt et al., 2018). Taken together, these findings suggest reduction of muscle fiber excitability may be the mechanism for NMJ transmission and action potential failure in very old mice noted on SFEMG. Future studies will need to be designed to investigate muscle fiber excitability parameters to determine whether this is the case. Our NMJ studies were performed in the gastrocnemius muscle (in vivo) and the tibialis anterior muscles (ex vivo), both of which are made up of primarily fast twitch muscle fibers. Prior studies in humans and rodents have shown differential effects of aging on muscles of differing fiber types (Cacciaet al., 1979; Lexell et al., 1988; Miljkovicet al., 2015; Xu et al., 2017). Although it is not possible to selectively perform in vivo SFEMG from one muscle fiber type versus another, future studies could explore changes of NMJ transmission in muscles with different fiber type proportions.

Another aim of the current experiments was determination of the age when C57BL/6J mice show motor unit losses and alterations of NMJ transmission. The rationale for this investigation was the consideration that motor unit losses and reinnervation may explain secondary changes in NMJ transmission due to NMJ remodeling. Similar to our prior work, we showed that motor unit losses occur by about 20 months. Interestingly, SFEMG findings of increased jitter and blocking were not demonstrated until 27 months of age. Because motor unit losses occurred before the time point when jitter increased, a reasonable inference would be that denervation is a contributor to NMJ transmission failure. However, as neuromuscular transmission is never successful at denervated NMJs, denervated muscle fibers do not contribute to measures of jitter and intermittent block. We thus conclude that while denervation of muscle fibers is a contributor to sarcopenia, denervated muscle fibers

do not contribute to the changes noted on SFEMG in 27- and 29-month-old mice. The fact that NMJ failure appear very late in relation to motor unit loss implies that NMJ failure could be an independent phenomenon and may not involve the dying back mechanism as has been reported in motor neuron diseases (Dadon-Nachum et al., 2011; Fischer et al., 2004). Alternatively, loss of motor units at 20 months of age may initiate sufficient compensatory response of reinnervation through collateral sprouting resulting in the absence of obvious NMJ transmission defects until after 24 months of age. At older ages, motor unit degeneration may supersede the regenerative response resulting in abnormal remodeling of the NMJ and abnormal NMJ transmission at 27 months of age. The correlation between MUNE and SFEMG blocking in 27- and 29-month-old mice would seem to argue in favor of the later possibility, but if motor unit degeneration and secondary NMJ failure were the cause of SFEMG blocking, EPC amplitude would be expected to be reduced which was not the case. Furthermore, an electrophysiological hallmark of early, immature reinnervation is reduced quantal content which was not overtly observed during our ex vivo NMJ studies (Katz et al., 1996). Understanding the pathophysiological events that result in the abrupt failure of NMJ transmission in 27-month-old mice requires future study. Within the groups of the 27- and 29-month-old mice, there was striking variability in the SFEMG measurements with some mice demonstrating jitter values similar to that in the younger cohorts. Such variability was also shown in gene expression changes in 27- and 29-month-old mice consistent with overall decreased control of gene regulation at very old ages (Barns et al., 2014).

Similar to the motor unit loss, muscle strength also showed an earlier decline at 20 months of age at the time when there were no overt NMJ defects. This suggests that the early loss of muscle strength is not due to failure of NMJ transmission but may be related to other factors such as denervation or subsarcolemmal dysfunction possibly affecting mechanisms of calcium release from intracellular calcium stores. In fact, excitation-contraction uncoupling is well described in the context of age-dependent decline in specific muscle force (Delbono, 2002; Taylor et al., 2009). These studies show age-dependent changes in voltage-gated calcium channels $Ca_v1.1$ and $Ca_v1.2$ resulting in the loss of muscle-specific force during old age (Delbono, 2003; Taylor et al., 2009). In addition, there are increases in expression of sodium channel isoform, Nav1.5, indicative of denervation in adult skeletal muscle (Taylor et al., 2009; Wang et al., 2005).

What alterations occur at the NMJ in older human adults remains a controversial area. Owing to the inaccessibility of adequate motor point biopsies, investigation in this area is challenging and thus underexplored. One recent study investigated tissues harvested from patients undergoing lower limb amputations to demonstrate that NMJ morphology is remarkably unchanged across cohorts of individuals of differing ages (Jones et al., 2017). Yet, other studies have shown age-dependent morphological remodeling of NMJs in different sets of human muscle samples (Oda, 1984; Wokke et al., 1990). Of interest was a recent clinical study that demonstrated that older men with sarcopenia demonstrated reduced reinnervation capacity as compared with men without overt sarcopenia (Piasecki et al., 2016). In this same study, MUNE was not significantly different in sarcopenic men when compared with nonsarcopenic men and therefore may suggest that although motor unit loss is evident in old age, it may not be driving the sarcopenic phenotype per se but it may be the

failure to reinnervate that predispose the old population toward more sarcopenic phenotype. Two additional studies investigated reinnervation capacity by examining muscle biopsies, and these studies also suggested that high performing older adults have increased capacity for reinnervation based on morphological features of fiber grouping (Mosole et al., 2014; Sonjak et al., 2019). SFEMG is routinely used in neuromuscular clinics for the diagnosis of myasthenic disorders, and it is well established that jitter is increased in healthy older adults (Bromberg and Scott, 1994). Future clinical studies could leverage stimulated SFEMG to investigate the contribution of NMJ transmission failure in older adults affected by sarcopenia.

Establishing the timeline of NMJ transmission defects in relation to motor unit loss and loss of muscle contractility has potential implications in designing novel therapeutic interventions specific for different stages of sarcopenia. Our findings of late NMJ transmission defects imply that modulating NMJ at earlier stages of life may not have relevant functional consequences. Future studies involving the NMJ modulation of different presynaptic and postsynaptic components at different ages are clearly needed to better understand the contribution of neuromuscular transmission defects in the loss of physical function in the elderly.

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Fig. 1. Representative SFEMG traces showing jitter and blocking in NMJ synapses in C57BL/6J mice. (A–D) Representative single-fiber action potentials recorded at an axonal stimulation frequency of 10 Hz from gastrocnemius muscle in C57BL/6J mice with a sweep speed of 500 μ s and a sensitivity of 500 μ V. A healthy NMJ synapse with normal jitter and no blocking is shown in panels A (superimposed responses) and B (raster responses). An example of an abnormal synapse with increased jitter and blocking (arrow) is shown in panels C (superimposed responses) and D (raster responses). Abbreviations: SFEMG, single-fiber electromyography; NMJ, neuromuscular junction.

Fig. 2.

Increased SFEMG jitter and blocking in NMJ synapses in very old 27- to 29-month-old C57BL/6J mice. (A) Mean jitter and (B) percentage of blocking are increased significantly in 27- and 29-month-old mice compared with 12-month-old mice. Group sizes: 12 months, $n = 5$ males and 5 females; 20 months, $n = 5$ male and 5 females; 24 months, 5 males and 5 females; 27 months, $n = 5$ males, 6 females; and 29 months, $n = 6$ males and 3 females.

**** $p < 0.0001$. Abbreviations: SFEMG, single-fiber electromyography; NMJ, neuromuscular junction.

Fig. 3.

Synapses with blocking at 10 Hz ($n = 11$) demonstrated increased action potential blocking at higher stimulation frequencies of 20 Hz and 30 Hz. (A–C) Example of synapse with rate-dependent increased blocking. Representative traces are shown with a sweep speed of 500 μ s and a sensitivity of 1 mV. (D) Comparison of blocking at 10 Hz, 20 Hz, and 30 Hz. At 10 Hz, there was 52% blocking (584/1121 stimulations), at 20 Hz, there was 63% blocking (715/1126 stimulations), and at 30 Hz, there was 69% blocking (796/1154 stimulations) at 30 Hz (chi-square, $p < 0.0001$). Group size: ($n = 13$, 9 males and 4 females) 21–30 months of age.

Fig. 4.

Age-dependent loss of motor unit numbers, decline in plantar flexion muscle torque, and increased frailty in C57BL6/J mice. (A) Triceps surae MUNE and (B) normalized plantar flexion tetanic torque are reduced, and (C) frailty index is increased in 20-, 24-, 27-, and 29-month-old mice when compared to 12-month-old mice. Group sizes: 12 months, $n = 5$ males and 5 females; 20 months, $n = 5$ male and 5 females; 24 months, 5 males and 5 females; 27 months, 5 males and 6 females; and 29 months, $n = 6$ males and 3 females. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 5.

Associations between in vivo measures and endpoint muscle mass in 27- and 29-month-old C57BL/6J mice. Heatmap of associations between mean SFEMG jitter (jitter), SFEMG blocking (blocking %), motor unit number estimation (MUNE), tetanic plantar flexion torque normalized to body mass (tetanic), frailty index (frailty), and triceps surae wet muscle weight (triceps surae) in 27- and 29-month-old mice. Group sizes: 27 months, n = 5 males and 6 females and 29 months, n = 6 males and 3 females. Spearman correlation coefficients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: SFEMG, single-fiber electromyography.

Fig. 6.

Ex vivo NMJ electrophysiology of the tibialis anterior muscle. Comparison of 23- and 29-month-old male mice demonstrated significant differences in the (A) miniature endplate current (MEPC) amplitudes ($p < 0.0001$), (B) frequency of MEPCs ($p = 0.027$), and (C) quantal content ($p = 0.002$), but this resulted in no significant differences in (D) EPC amplitude ($p = 0.816$). Group sizes: A total of 73 synapses were studied from 3 male mice aged 23 months, and a total of 100 synapses were studied from 4 male mice aged 29 months. Data shown as mean $\pm$ standard deviation. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. Abbreviations: NMJ, neuromuscular junction; EPC, endplate current.

Table 1:

In vivo and ex vivo measurements and respective gender-specific group sizes

Measurement	Group size	Nerve and location of stimulation	Muscle(s)	Parameters
In vivo muscle contractility	4–6 males and 4–6 females at each age	Tibial nerve at the medial knee	Tibial innervated plantar flexor muscles	Maximum torque during a train of stimulation at 150 Hz
In vivo electrophysiology	4–6 males and 4–6 females at each age	Sciatic nerve at the proximal hindlimb	Gastrocnemius and soleus	Motor unit number estimation
Single-fiber electromyography (SFEMG)	4–6 males and 4–6 females at each age	Sciatic nerve at the proximal hindlimb	Gastrocnemius	Single muscle fiber action potential jitter and blocking
Frequency dependency SFEMG	9 males and 4 females (ages 21–30)	Sciatic nerve at the proximal hindlimb	Gastrocnemius	Single muscle fiber action potential jitter and blocking
Ex vivo neuromuscular junction electrophysiology	3 males at 23 mo and 4 males at 29 mo	Terminal twigs of the fibular nerve	Tibialis anterior	Miniature endplate potential current and frequency, endplate current, and quantal content