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Enhancement of neuromuscular dynamics and strength behavior using extremely low magnitude mechanical signals in mice

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Abstract

Exercise in general, and mechanical signals in particular, help ameliorate the neuromuscular symptoms of aging and possibly other neurodegenerative disorders by enhancing muscle function. To better understand the salutary mechanisms of such physical stimuli, we evaluated the potential for low intensity mechanical signals to promote enhanced muscle dynamics. The effects of daily brief periods of low intensity vibration (LIV) on neuromuscular functions and behavioral correlates were assessed in mice. Physiological analysis revealed that LIV increased isometric force production in semitendinosus skeletal muscle. This effect was evident in both young and old mice. Isometric force recordings also showed that LIV reduced the fatiguing effects of intensive synaptic muscle stimulation. Furthermore, LIV increased evoked neurotransmitter release at neuromuscular synapses but had no effect on spontaneous end plate potential amplitude or frequency. In behavioral studies, LIV increased mouse grip strength and potentiated initial motor activity in a novel environment. These results provide evidence for the efficacy of LIV in producing changes in the neuromuscular system that translate into performance gains at a behavioral scale.

Keywords

Whole body vibration; neuromuscular; quantal content; muscle strength; mechanical signals

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CONFLICT OF INTEREST STATEMENT

Clinton T. Rubin serves as Chief Scientific Officer for Marodyne Medical. All other authors have no conflict of interest, and all experiments were conducted independent of Dr. Rubin, who acted as an advisor and provided equipment for the LIV treatments.

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Numerous studies using Whole Body Vibration (WBV) of varying frequencies and magnitudes, or limb specific vibration, including LIV, suggest it can improve skeletal and muscular function. Specifically, high frequency, low magnitude vibration stimulates bone formation, and may suppress adipose tissue generation (Gilsanz et al., 2006; Holguin et al., 2009; Reyes et al., 2011; Rubin et al., 2007; Slatkowska et al., 2010; Xie et al., 2006; Xie et al., 2008). Additionally, improvements in muscular function have also been demonstrated in humans (Gilsanz et al., 2006; Muir et al., 2011; Reyes et al., 2011; Russo et al., 2003) and animals (McKeehen et al., 2012; Xie et al., 2008). However, as opposed to human studies, there have been few if any reports of the efficacy of WBV or LIV at the behavioral level in animals.

Here we examine the influence of brief daily exposure to LIV on the musculature of both young and old mice as a means of understanding the mechanisms by which these mechanical signals modulate muscle function. We describe a number of potentially beneficial effects using both physiological and behavioral approaches. An addition, we propose a partial mechanism for one aspect of the observed changes, which may help to explain conflicting results from this new field.

METHODS

Animals

Muscle electrophysiology, *ex vivo* isometric tension, and preceding LIV exposure were approved by the Institutional Animal Care and Use Committee and performed at the University of Missouri Dalton Cardiovascular Research Center. For *ex vivo* isometric tension and intracellular recording experiments, male C57BL/6 mice of 8 and 104 weeks of age, with $n = 4$ and $n = 3$, respectively, were subjected to LIV every day for 4 weeks and sacrificed 2 to 3 days after the last exposure. LIV exposure was performed for 20 min each day with mice in empty cages on a custom manufactured platform oscillating vertically at 30 Hz with 0.3 g peak acceleration (Marodyne Medical, Lakeland, FL). Control mice were set in identical housing near the platform during LIV exposure.

Behavioral experiments and preceding LIV exposure were approved by the Institutional Animal Care and Use Committee and performed at The University of Texas Southwestern Medical Center. Behavioral experiments were performed during the light cycle under dimmed lighting. Male C57BL/6J mice were purchased from Jackson Laboratory at 8 weeks of age and allowed to acclimate to housing for one week. Mice were group housed with 4 per cage. Half of the mice (two from each cage) were assigned as the LIV treatment group ($n = 12$) with the other half being controls. Exposure was performed as above at 5 times per week for 3 weeks before behavioral testing and continued one more week, after daily testing. Behavioral testing, in order, consisted of Wire Grid Hang, Wire Hang, Grip Strength, and Open Field.

Ex vivo Isometric Tension and Electromyogram Measurements

These experiments were performed as previously described (Rafuse et al., 2000). Briefly, mice were sacrificed by CO₂ asphyxiation and semitendinosus muscle with intact nerve supply was dissected out and placed into well-oxygenated Tyrode's solution maintained at 27–29°C. Fine-tipped polyethyl suction electrodes were used to stimulate individual nerves and measure electromyograms from the midbelly of the muscle. A linear force transducer was used to measure muscle contractions. Nerves were stimulated repeatedly 4–5 times at frequencies of 1, 10, 20, 50, 100, and 200 Hz. The average peak force from each stimulation set was used in statistical testing.

Intracellular Recordings

Intracellular recordings were also performed as previously described (Rafuse et al., 2000). Semitendinosus muscles, with the nerve supply intact, were isolated as described above. Miniature end plate potentials (MEPPs) and evoked end plate potentials (EPPs) were recorded using sharp glass electrodes impaled near the motor end plate under standard conditions. The quantal content per cell was calculated by the mean amplitude of the first EPP from all the train stimuli (from 10 to 200 Hz) divided by the mean amplitude of the MEPPs from the same cell recording. Recording in which MEPPs were not observed at the same time that EPPs from the same recording cell were not used in the calculations. The mean quantal content was calculated as the mean of all the individual QCs from the control and treated animals.

Neuromuscular junction staining

Post electrical evaluation, the muscle preparations were incubated with an α -bungarotoxin-based stain (α -BTX-Alexa 555, Invitrogen, 100 nM) for 15 min in oxygenated Tyrode's solutions. After 15 min the preparation was washed 3 times with new fresh Tyrode's solution. The preparation was maintained under oxygenation and imaged in a fluorescence Olympus BX51WI microscope with a 40x water immersion lens. Images were captured by a Retiga EXi-Fast (Qimaging) in binning mode at 8 bits.

Behavioral Experiments

Wire Grid Hang—Mice were placed first on a thin wire grid and then a wire rat cage grid as tests of muscle strength, endurance, and coordination (Hamann et al., 2003; Lee et al., 2009; Sango et al., 1996). The grid was shaken lightly to encourage mice to grip, then flipped upside down and the latency for mice to fall was recorded using a stopwatch. One trial was performed for each mouse with each grid.

Wire Hang—This test was used as another measure of muscle dynamics (Allen et al., 2009; Baldo et al., 2012; Oddoux et al., 2009; Takahashi et al., 2009). Mice were lifted by the tail and allowed to grip onto a horizontal bar (diameter = 3.175mm) with their forepaws, then let go and allowed to hang until falling a short distance. All mice tested in this way were able to lift their lower bodies to grip the bar with all four limbs, as well as the tail. Two trials of wire hanging were performed at separate times on one testing day and averaged for statistical testing.

Grip Strength—As a final measure of muscle strength (Derave et al., 2003; Mandillo et al., 2008; Whittemore et al., 2003), mice were placed on a horizontal wire grid attached to a spring loaded linear scale and allowed to grip it with all four paws, then were pulled by the tail until grip was released. The final weight pulled was then recorded. Three trials of this test were performed on each of 3 days. The average of each day's 3 trials was used for statistical testing.

Open Field

This paradigm was used to test for effects of vibration on locomotion as well as general anxiety (Crawley, 1999). Mice were placed in a dimly lit square arena with walls 44 cm in length as a novel open field. Individual trials were 20 min in length, recorded using an overhead camera and analyzed using EthoVision v. 3.1.16. Measures in this study included total distance moved per arena region, distance moved per 4 min epoch, and total duration within specific regions of the testing arena.

Statistical Testing

Two-way partial repeated measures ANOVA were performed on time series data from body mass and open field measures. Post-testing was performed using the Sidak method to correct for multiple comparisons. Two-way ANOVA was performed on *ex vivo* force production data. For other data, unpaired Student's *t*-tests were performed. Values of *p* less than 0.05 were considered to be significant.

RESULTS

To understand how LIV could improve neuromuscular performance, we first used a physiological approach. Adult or aged mice were either left untreated (controls) or treated long term with LIV. From these subjects, *ex vivo* semitendinosus muscle preparations were then made for assessment of isometric force production in response to increasing synaptic stimulation rates (Fig. 1). In both young and old mice, LIV treatment resulted in overall higher isometric force (both $p < 0.0001$). At higher stimulation rates, where untreated mouse muscle ceased to produce considerable force, LIV mice demonstrated continued, although reduced, force generation in young (Fig. 1A) and old (Fig. 1B) mice. In young mice there were significant increases at 20, 100, and 200 Hz stimulation rates. These were higher by 1.6-, 10-, and 29-fold, respectively. In aged mice, the effect was significant only at 100 and 200 Hz ($p = 0.0020$ and 0.0055 , respectively) with increases of 3.5- and 7-fold.

The assessment of LIV's effects on overall strength were derived from average peak force produced by from 4–5 pulse trains with 1 sec intervals, of increasing frequency. Examination of individual traces provides additional information regarding response kinetics (Fig. 2). For example, muscles exhibited a temporal decrease in peak force upon successive stimulations. This may be attributable to muscle fatigue, which was attenuated by LIV. Together these data indicate that LIV enhances neuromuscular function in both young and aged mice.

To better understand the effects of LIV on muscle strength, neuromuscular synapse function was examined electrophysiologically. Spontaneous release of neurotransmitter vesicles was assessed by recording miniature excitatory endplate potentials (MEPP). LIV had no detectible effect on either MEPP amplitude or frequency (Fig. 3A and B). In contrast to spontaneous activity, evoked endplate potentials from LIV treated mice exhibited a significant increase in quantal content (1.2-fold), compared to controls (fig. 3C). Immunostaining of endplates revealed no differences in size or surface area (Fig. 3D). These data suggest a greater number of synaptic vesicle release events per action potential.

To determine how the LIV-induced physiological effects translated into functional outcomes, a series of behavioral tasks dependent upon neuromuscular function were assessed. As an initial assessment there was an approximately 4% reduction of 3 week LIV treated mouse body mass compared to controls. This effect was not statistically significant, and was in agreement with another report (Rubin et al., 2007). To test neuromuscular function in mice, we chose three commonly used protocols, Wire Grid Hang, Wire Hang, and Grip Strength tasks. Each of these tasks involve exertion of limb muscle strength. In both the Wire Grid Hang and Wire Hang tests no significant effects of LIV treatment were detected. However, there was a significant increase in measured Grip Strength (1.1-fold) on the first day of the test (Fig. 4). This effect did not persist through the second and third days of the test, possibly due to task habituation.

Locomotor activity is also dependent upon neuromuscular junction behavior and declines with age. To assess the effect of LIV on this basic behavior, control and treated mice were placed in a novel open field for 20 min (Fig. 5). Although no overall differences in activity

were seen between groups, there was a significant interaction effect ($p = 0.0003$). Post-testing revealed that treated mice moved a significantly greater distance (1.2-fold, $p = 0.0257$) than controls during the first 4 min epoch. The increased initial activity occurred in the periphery, where mice are typically most active during initial exploration. There was no significant change in time spent in the middle of the open field, suggesting no effects on basal states of anxiety or mood.

Together these behavioral studies demonstrate that LIV-induced changes in neuromuscular physiology correlate with significant effects in the performance of behavioral tasks in which neuromuscular function is a critical component.

DISCUSSION

While there have been a variety of human studies attempting to show muscle performance gains from WBV, there has been some debate as to the treatment's efficacy (for a review see ref. 28). Differences in treatment parameters and performance criteria further prohibit useful comparison and interpretation of published results. Here we demonstrate modification of neuromuscular system function by LIV in a mouse model by *ex vivo* muscle electrophysiology and *in vivo* animal behavior. While the mechanistic basis by which mechanical stimulation achieves these effects remains to be further delineated, our findings suggest that reduction of fatigability, increased muscle strength, and/or increased quantal content at the neuromuscular junction represent three potentially independent, or more likely interdependent effects of LIV on muscle performance.

Regarding muscle fatigability, one of the major determinants of the course of adult muscle adaptation is the frequency of motor neuron stimulation they receive, though there is some evidence that myoblasts may have intrinsic anabolic sensitivity to vibrational stimuli (Wang et al., 2010). Muscles can be influenced by chronic stimulation paradigms to alter their metabolic, myosin heavy chain, and myosin light chain phenotypes within muscle-specific adaptive ranges (Bozzo et al., 2005; Gorza et al., 1988; Westgaard and Lomo, 1988; Windisch et al., 1998). Such changes are characterized by a succession of expression patterns. Responses to chronic low frequency stimulation in muscle include increased expression and activity of enzymes that mediate oxidative metabolism, followed by alterations of the dominant myosin heavy chain (Jaschinski et al., 1998; Takahashi and Hood, 1993). These changes coincide with increased endurance of stimulated muscle (Takahashi and Hood, 1993; Westgaard and Lomo, 1988). Such endurance changes mirror those seen in rodent models of endurance training (Carter et al., 1995; Jeneson et al., 2007; Rouviere et al., 2012), not only by *in vivo* measures of muscle fatigability, but also increased oxidative metabolic enzyme concentrations.

More speculatively, LIV could affect muscle type transformation through a mechanism that converts the vibratory stimulus into descending motor impulses. The H-reflex pathway, a monosynaptic excitatory influence from intrafusal afferents upon *alpha* motor neurons, could provide one such mechanism. Vibration in the frequency used in our experiments elicits frequency-matched Ia and II afferent firing *in vitro* using frog (Querfurth, 1985) and cat (Hunt and Ottoson, 1977) preparations. Other experiments have shown *in vivo* activation of this pathway in cats (Kroller et al., 1988) and humans (Burke et al., 1976). Notably, in cats, for lower vibration frequencies, between 2 and 20 Hz, there is significant amplification of excitatory afferent input to *alpha* motor neurons (Powers et al., 2012). Furthermore, mouse lumbar *alpha* motor neurons exhibit resonance frequencies for excitation between 7 and 30 Hz (Manuel et al., 2009). Frequency-matched stimulus-driven *alpha* motor neuron activity has also been demonstrated in humans using vibratory stimuli (Fornari and Kohn, 2008; Person and Kozhina, 1992; Tsang et al., 2008), though at lower amplitudes such

stimulation may not be effective (Fallon and Macefield, 2007). Thus it is possible that LIV, at low frequencies, elicits increased *alpha* motor neuron firing at those same frequencies and, consequently, slowly induces changes in muscle towards a slower oxidative, fatigue-resistant phenotype.

As a second consideration, LIV may improve muscle strength. Peak isometric force is proportional to cross sectional area, if contractile protein concentration and cross bridge density are constants (Fitts et al., 1991). WBV at a variety of frequencies has been shown to induce slight increases muscle cross sectional area (Gilsanz et al., 2006; Xie et al., 2008), although other studies report only a non-significant trend (McKeehen et al., 2012; Murfee et al., 2005). Other measures of strength have also demonstrated WBV efficacy in increasing muscle power (Muir et al., 2011; Russo et al., 2003), isometric force (McKeehen et al., 2012), and torque in some muscles (Rees et al., 2007; Rees et al., 2008). The mechanisms by which normal exercise induces muscle hypertrophy are multifactorial, including hormonal, loading, and neuromuscular influences upon a number of so far discovered genetic pathways (for a short review see ref. 44) and are still not well understood. How LIV may influence these mechanisms is not yet clear, although the data presented here are at least consistent with the possibility that LIV may cause muscle hypertrophy.

As a third component, LIV increased the quantal content of neurotransmitter release at the mouse neuromuscular junction. Few if any studies have heretofore assessed the effects of vibration on quantal content. Studies of neuromuscular junction quantal content are somewhat conflicting, particularly with regard to muscle type. For example, it has been suggested to be higher in slow versus fast muscles (Everett and Ernst, 2004; Taquahashi et al., 1999), while at least one other report indicates that there are no difference between muscle types (Edwards et al., 1998). Additionally, exercise has been found to increase quantal content specifically for fast but not slow muscle (Dorlochter et al., 1991; Taquahashi et al., 1999), while also increasing succinate dehydrogenase (a marker for mitochondrial oxidative metabolism) staining in low oxidative fibers (Dorlochter et al., 1991). Consequently, it is possible that the LIV-induced increases in quantal content reported here indicate of the beginnings of muscle fiber type transformations.

The physiological effects of LIV correlated with changes in mouse behaviors dependent upon the neuromuscular system. While the increase in initial grip strength in LIV-treated mice is consistent with improved muscle performance, there were no overt indications of this in other measures of strength or mobility. Grip Strength and Wire Grid Hang tests are included in widely used and validated phenotyping protocols (Crawley, 1999; Mandillo et al., 2008). However the Wire Hang test is less standardized, with differing implementations and interpretations (Allen et al., 2009; Alonso et al., 2008; Baldo et al., 2012; Oddoux et al., 2009; Takahashi et al., 2009). In the paradigm used here, LIV-induced mice showed no improvement. However, all mice were able to grip the single wire with all four limbs plus tail. Thus it is possible that the results were affected by factors other than strength, such that differences of strength would have had relatively small effects.

LIV increased initial motor activity upon exposure to a novel environment. Though this change may be related to alterations in neuromuscular physiology, caution must be used in any specific interpretation without a more thorough investigation of mechanisms. Our behavioral studies were conducted in normal healthy adult mice. It would also be interesting to assess how LIV could affect the recovery, outcome, or progression of animal models of diseases affecting neuromuscular function.

In summary, in addition to the effects of LIV on the musculoskeletal system (Gilsanz et al., 2006; Reyes et al., 2011; Xie et al., 2006; Xie et al., 2008), the effects reported here are

consistent with the possibility that LIV stimulates at least a partial transformation of fast muscle towards a slower oxidative phenotype and may induce small amounts of muscle hypertrophy. These effects resemble changes induced in mice by exercise and may involve similar mechanisms. Further experimentation would be required to test this hypothesis. If true, this brief, non-strenuous passive exposure to low intensity vibrations could represent the early stages of an intervention which targets populations suffering from neuromuscular or musculoskeletal disorders by enabling improvements in both bone health and muscle dynamics.

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Fig. 1.

LIV increases isometric force production and enhances neuromuscular synapse-driven responsiveness in mouse skeletal muscle. Effects of 4 weeks of LIV exposure on the isometric force generation of isolated mouse semitendinosus muscle at varying frequencies of intact direct nerve stimulation from young (12 week old, $n = 4$, A) and aged (107 week old, $n = 2-3$, B) mice are summarized. LIV increased force production overall in young and old mice ($p < 0.0001$ for both age groups). Data represent means with standard error. For per frequency comparisons **** is $p < 0.0001$ and ** is $p < 0.01$.

Fig. 2. Isometric force recordings show fatigue of synapse-driven muscle responses is reduced and the threshold for loss of responsiveness is raised by LIV. Two representative isometric force traces from control (A) LIV exposed (B) mice are depicted. Note that control muscle responded with less peak force at stimulation rates above 10 Hz and ceases to respond significantly above 50 Hz.

Fig. 3.

LIV increased evoked neurotransmitter release at neuromuscular synapses but had no effect on miniature end plate potential (MEPP) amplitude or frequency. Histograms for MEPP amplitudes (A), frequency (B), and calculated quantal content (C) measured electrophysiologically in LIV-exposed and control 12 week-old mouse semitendinosus neuromuscular junctions with accompanying example recording traces are shown. Data represent means with standard error (* $p = 0.0336$, $n = 198-244$). (D) Immunostains of neuromuscular junctions from control and LIV-treated mouse semitendinosus muscle.

Fig. 4. LIV increases mouse grip strength. Quantitation of grip strength of LIV exposed and control 12 week-old mice is shown. Data represent means with standard error (** $p = 0.0097$, $n = 12$).

Fig. 5. LIV potentiates initial motor activity in a novel environment. Plots of distance moved in an open field in 4 min bins are shown for LIV exposed and control 12 week old mice. Data represent means with standard error (* $p = 0.0257$, $n = 12$).