Prevention of Age-related Neuromuscular Junction Degeneration in Sarcopenia by Low-magnitude High-frequency Vibration

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Abstract

Neuromuscular junction (NMJ) degeneration is one of the pathological factors of sarcopenia. Low-magnitude high-frequency vibration (LMHFV) was previously reported effective in alleviating the progress of sarcopenia in SAMP8 mice. In this study, we observed more acetylcholine receptors (AChRs) branching in sarcopenic NMJ in humans. Utilizing a validated non-sarcopenic (SAMR1) versus sarcopenic (SAMP8) animal model, NMJ degeneration was observed to precede the onset of sarcopenia where the SAMR1 mice presented better NMJ function and more intact NMJ structure. After the treatment of LMHFV in SAMP8, progress of sarcopenia and NMJ degeneration were both alleviated with increased Dok7 expression, one protein important for NMJ assembly and function. In vitro, when Dok7 expression was knocked down, myotube diameter and acetylcholine receptors (AChRs) cluster formation were decreased, that could not be retarded by LMHFV. Further mechanistic investigation showed that LMHFV could suppress ERK1/2 activation and the blockade of ERK1/2 phosphorylation promoted AChRs clustering and increased Dok7 expression. In myotubes with ERK1/2 phosphorylation blocked, knocking down Dok7 could still reduce AChRs cluster formation, which could not be retarded by LMHFV. Therefore, effectiveness of LMHFV on Dok7 expression in NMJ degeneration was shown to act through suppressing ERK1/2 phosphorylation.

Introduction

Sarcopenia is a geriatric syndrome characterized by progressive decrease of muscle mass and strength, which will lead to reduced physical performance and poor quality of life in older people \(^1\). The etiology of sarcopenia is multi-factorial with the degeneration of neuromuscular junction (NMJ) being one of the major causes \(^2,3\). NMJ is a chemical synapse serving as a bridge between a motor neuron and a muscle fiber where the major function is to convert action potentials in the motor neurons into chemical signals across the synaptic cleft to trigger muscle contraction \(^4,5\). Ageing related changes in the NMJ have been well reported to show morphological changes (e.g. endplate fragmentation) and functional adaptations \(^6\--^8\). The maintenance of endplate morphology during ageing is affected by acetylcholine receptors (AChRs) clustering at the post-synaptic cleft on the muscle side initiated by the large proteoglycan, agrin, released at the pre-synaptic nerve endings \(^8\). Across to the muscle side, agrin binds to the low-density lipoprotein receptor-related protein 4 (LRP4) to trigger the auto-phosphorylation of the muscle-specific kinase (MuSK) along with the substrate of docking protein-7 (Dok7) that eventually leads to the clustering of AChRs through the receptor-associated protein of the synapse (Rapsyn) \(^4,9,10\).

Low-magnitude high-frequency vibration treatment (LMHFV, 35Hz, 0.3g) is a non-pharmaceutical treatment modality that can be regarded as a passive weight-bearing exercise. It was reported effective clinically in reducing falls and fracture incidences and enhancing muscle performance in the community dwelling older people \(^11\). This modality was also reported effective in maintaining skeletal muscle function in sarcopenic animal model \(^11\--^13\) with evidence to target other pathological factors like
intramuscular fat infiltration or adipogenesis\textsuperscript{14,15}. However, there is no previous study investigating the treatment effects of LMHFV specifically targeting denervation in sarcopenia, or the degeneration of NMJ.

In this study, we systematically characterized the age-related degeneration of the NMJ morphologically and functionally in a well-accepted sarcopenic animal model\textsuperscript{13}, the senescence accelerated prone-8 mice (SAMP8) against its control strain, the senescence accelerated mouse resistant-1 (SAMR1) mice developed by Takeda et al. at the Kyoto University\textsuperscript{13,16}. Here, we show that denervation preceded the onset of sarcopenia and vibration therapy was effective in reversing this progress. We further investigated the treatment effect of LMHFV therapy on the maintenance of the NMJ during the onset of sarcopenia with respect to the Agrin-LRP4-MuSK-Dok7 pathway and looked into the mechanism to prove that vibration therapy enhanced AChRs clustering by increasing Dok7 expressions in the Agrin-LRP4-MuSK-Dok7 structural complex through mechanically-induced inhibition of extracellular signal-regulated kinase (ERK)1/2 signaling.

**Results**

**Deterioration of NMJ morphology in sarcopenic versus non-sarcopenic human subjects**

NMJ morphology was previously reported to be independent of ageing\textsuperscript{17}, but no study has ever reported the difference in NMJ morphology between sarcopenic and non-sarcopenic patients. To confirm the relationship between NMJ deterioration and sarcopenia, we collected vastus lateralis muscle biopsy of hip fracture patients with and without sarcopenia. Sarcopenic patients (83.67 ± 8.5 year) presented reduced grip strength (12.33 ± 2.08N vs 21.00 ± 7.07N) and appendicular skeletal muscle mass index (ASMI) (4.84 ± 0.11kg/m\textsuperscript{2} vs 5.97 ± 1.07 kg/m\textsuperscript{2}) than non-sarcopenic patients (76.00 ± 9.9 year) (Table. S1).

Human NMJs in sarcopenic patients showed smaller terminal axons, more rudimentary nerve terminals, smaller and distinctive “nummular” (coin-shaped patches) endplates (Fig. 1A). Quantitatively, sarcopenic NMJ presented more AChRs branching number than non-sarcopenic NMJ (p < 0.001, Fig. 1A and C), while AChRs cluster fragmentation, area, the compactness of AChRs cluster and endplate area showed no significant differences between sarcopenic and non-sarcopenic NMJ (Fig. 1B, D and E). Taken together, development of sarcopenia clearly showed a difference in NMJ morphology in humans that requires further investigation in terms of morphology and functions.

**Skeletal muscle deterioration in sarcopenic mice model is suppressed by LMHFV**

In order to investigate NMJ degeneration in relation to the onset of sarcopenia, a well-accepted sarcopenic animal model, SAMP8 against its control strain, SAMR1 without sarcopenia phenotype were used in this study\textsuperscript{13,18}. The animal model is validated functionally and morphologically along with
molecular evidence of muscle atrophy. As grip strength test is clinically the most common method to assess muscle strength, grip strength is usually used in animal studies to evaluate the motor performance in vivo because the neuromuscular system will be recruited during the functional test \(^\text{19}\). In SAMP8 mice, grip strength decreased significantly by 27.84\%, 23.46\% and 32.00\% at 10 months compared with 3, 6 and 8 months old (p < 0.001, Fig. 2A). In SAMR1 mice, grip strength also showed significant 12.77\% and 28.73\% decrease at 10 months compared with 3 and 6 months old (p < 0.05 and < 0.01 respectively, Fig. 2A), but SAMR1 presented significantly 14.1\% and 27.4\% higher grip strength than SAMP8 mice at 6 and 10 months, respectively (p < 0.01 for both, Fig. 2A).

We further employed \textit{ex-vivo} electrophysiology assessments to investigate the age-related changes of muscle function in SAMP8 and SAMR1 mice at the triceps surae muscles. Tetanic force of SAMP8 mice increased from 3 months and peaked at 8 months compared with 3-month-old followed by a significant decrease at 10 months (p < 0.05 and < 0.01 respectively, Fig. 2B). SAMR1 mice showed relatively more stable tetanic forces during ageing. At 3 and 10 months old, SAMR1 mice presented 24.3\% and 22.9\% higher tetanic force compared with SAMP8 (p < 0.01 and < 0.05 respectively, Fig. 2B).

Dual energy X-ray absorptiometry (DXA) is the most common method to assess muscle mass in both clinical and pre-clinical studies but it requires animals under general anesthesia during the test \(^1\,\text{15}\). To minimize synaptic transmission and preserve NMJ function for further \textit{ex vivo} NMJ function test, it is necessary to shorten the anesthesia time to the most extent. As a result, muscle wet weight and cross-sectional area (CSA) were chosen to assess muscle mass in this study. Muscle mass increased from 6 to 8 months but dropped significantly by 19.51\% of wet weight and 15.63\% of CSA at 10 months in the SAMP8 (wet weight: p < 0.05 and < 0.01 respectively, CSA: p < 0.05 and < 0.05 respectively, Fig. 2C \text{and} D). At 3, 6 and 10 months, SAMR1 presented larger muscle mass than those of SAMP8 (wet weight: p < 0.01, < 0.01 and < 0.05 respectively, CSA: p < 0.01, < 0.001 and < 0.05 respectively, Fig. 2C \text{and} D). Furthermore, mRNA levels of muscle atrophy markers, Atrogin-1 and MuRF-1, were significantly higher in SAMP8 compared with SAMR1 at 10 months (p < 0.01 and < 0.05 respectively, Fig. 2E).

Our previous study reported that SAMP8 exhibited a higher proportion of type IIa but lower proportion of type IIb fibers with increasing age from 6 to 10 months old \(^18\). In this study, SAMR1 non-sarcopenic strain was also shown to exhibit a significantly 63.69\% lower proportion of type I but 41.23\% higher proportion of type IIb fibers than those of SAMP8 mice at 10 months old (p < 0.001 and < 0.05 respectively, Fig. 2F \text{and} G). Taken together, sarcopenic phenotype of SAMP8 is confirmed at 10 months of age against its SAMR1 control strain confirmed in terms of muscle function, morphology and muscle atrophy gene expressions. This important feature is used to compare occurrence of NMJ deterioration in later part of the study.

As a mature and ready-to-translate biophysical intervention, we provided LMHFV treatment as a mechanical cue to investigate the effect on sarcopenia in relations to NMJ degeneration. Because isometric contraction is generated when receiving vibration treatment and the mice are quadrupedal \(^20\), forelimb grip strength was used to assess neuromuscular function and \textit{ex vivo} hindlimb muscle isometric
force was used to evaluate muscle strength. At month 4 post-treatment, both grip strength and tetanic
force of the triceps surae were elevated in VIB group than CTL group (p < 0.05 and < 0.01 respectively,
Fig. 2H and I), indicating the neuromuscular function was improved. Although LMHFV presented no
significant effects on the triceps surae muscle mass at month 2, 4 and 6 post-treatment (Fig. 2J and K),
VIB group showed significantly reduced mRNA expressions of Atrogin-1 and MuRF-1 at month 4 post-
treatment (p < 0.05 and < 0.05 respectively, Fig. 2L). Furthermore, LMHFV could significantly reduce type I
fiber distribution by 40.13% but increase type IIb fiber composition by 62.71% at month 4 post-treatment
(p < 0.01 and < 0.05 respectively, Fig. 2M and N). Taken together, LMHFV could significantly improve
muscle quality in sarcopenic SAMP8 but presented no significant effects on muscle quantity.

Functional degeneration of NMJ preceded the onset of sarcopenia during ageing in sarcopenic SAMP8 mice

To investigate if NMJ degeneration is associated with the onset of sarcopenia, that is previously reported
to occur at 10 months in the SAMP8 sarcopenic mice versus the SAMR1 non-sarcopenic control, NMJ
function was further investigated. Employing ex vivo electrophysiology, 2 tetanic stimulations of
consecutive 100 cycles with a resting time of 0.7s each were given directly to muscle or the sciatic nerve
at 50 Hz to investigate the NMJ functional characteristics. At 10 months, SAMP8 presented significantly
increased neurotransmission failure than SAMR1 (Fig. 3A). In SAMP8 with increasing age,
neurotransmission failure was significantly increased at 8 months compared with 6 months (Fig. 3B), yet
no significant difference was found between 3 and 6 months (Fig. S3A) or 8 to 12 months (Fig. S3B),
indicating a remarkable deterioration of neurotransmission at 8 months in SAMP8 mice. Intra-tetanic
fatigue was another parameter assessing NMJ function and given by the percentage force at the end of
every 10 stimuli compared to the maximum force generated during that same pulse train. SAMP8 at 8
months old presented significantly reduced intra-tetanic fatigue from 20th to 40th stimulus of direct
muscle stimulations compared with 6 months (p < 0.05, Fig. 3C), but after nerve stimulations, intra-tetanic
fatigue at 8 months tended to be increased compared with 6 months, with significant difference at 40th
stimulus (p < 0.05, Fig. 3D). Furthermore, from 3 to 6 months and from 8 to 12 months old, no significant
differences were observed in intra-tetanic fatigue of both muscle and nerve stimulations in SAMP8 mice
(Fig. S3C, D, E and F). These results suggested significant NMJ fatigue occurs at 8 months in SAMP8
mice.

To further investigate the mechanisms of NMJ functional degeneration in SAMP8, acetylcholinesterase
(AChE) activity was measured. AChE is located on the post-synaptic membrane of NMJ and its major
function is to inactivate acetylcholine (ACh), thereby preventing sustained depolarization of post-synaptic
membrane 21. AChE activity significantly increased from 3 to 6 months followed by a significant
reduction at 8 months compared with 6 months (p < 0.05 for both, Fig. 3F). This could explain the
increased relative half relaxation time (1/2 RT) between nerve and muscle stimulations in tetanic pulse
trainings from 6 to 8 months in SAMP8 mice (p < 0.01, Fig. 3E), indicating longer time was required by the
NMJ to recover from the tetanic stimulations.
AChRs are formed by five subunits in which \( \alpha \) and \( \delta \) subunits constitute the binding site with ACh \(^{22}\). Like the age-related changes of AChE activity, mRNA expressions of both \( \alpha \) and \( \delta \) subunits in SAMP8 mice were significantly increased from 3 to 6 months, followed by a reduction at 8 months compared with 6 months (\( \alpha \) subunit: \( p < 0.05 \) and \( < 0.01 \) respectively, \( \delta \) subunit: \( p < 0.05 \) and \( < 0.05 \) respectively, Fig. 3G and H). Therefore, functional degeneration of NMJ was observed with a significant drop of AChRs structural components and AChE activities in the sarcopenic SAMP8 mice at 8 months of age that preceded the confirmation of sarcopenia (in terms of muscle function and morphology) at 10 months of age.

**LMHFV attenuated NMJ degeneration in sarcopenic SAMP8 mice**

To further investigate the treatment effect of LMHFV on the preservation of NMJ function during the onset of sarcopenia in SAMP8, vibration was given to the sarcopenic SAMP8 mice at 6 months of age (before the onset of NMJ degeneration). It was found that VIB group presented significantly reduced neurotransmission failure at month 2 and 6 post-treatment, yet no difference at month 4 post-treatment (Fig. 4A, B and C). The assessment of NMJ fatigue revealed that LMHFV presented no significant effects on the intra-tetanic fatigue after direct muscle stimulations, but at month 2 and 6 post-treatment, intra-tetanic fatigue was significantly improved by 14.91% and 35.23% after nerve stimulations at 40th and 50th stimulus respectively (\( p < 0.01 \) and \( < 0.05 \) respectively, Fig. 4D, E and F), indicating LMHFV presented no significant effects on muscle fatigue but could significantly attenuate NMJ fatigue.

To further study the mechanisms of improved NMJ function, it was found that LMHFV could significantly reduce AChE activity at month 2, 4 and 6 post-treatment (\( p < 0.01 \), \( < 0.05 \) and \( < 0.05 \) respectively, Fig. 4H). This could be explained by that the neuromuscular system would be recruited to generate tetanic and isometric contraction force, while reduced AChE activity could ensure sufficient binding between ACh and AChRs. Furthermore, \( \alpha \) and \( \delta \) subunit expressions were significantly increased at month 2 post-treatment (\( p < 0.05 \) and \( < 0.01 \) respectively, Fig. 4I), altogether contributing to the binding between ACh and AChRs, thus resulting in improved NMJ function at month 2 post-treatment. Reduced AChE activity extended the relative 1/2 RT of nerve and muscle tetanic stimulations at month 4 post-treatment (\( p < 0.05 \), Fig. 4G). Besides, \( \alpha \) and \( \delta \) subunit expressions were also significantly reduced at month 4 post-treatment (\( p < 0.05 \) and \( < 0.05 \) respectively, Fig. 4I). All these results indicated that LMHFV treatment could alleviate NMJ functional degeneration in a short term after the treatment by reducing AChE activity and increasing \( \alpha \) and \( \delta \) subunit expression.

**NMJ morphological degeneration preceded the onset of sarcopenia during ageing in sarcopenic SAMP8 mice**

NMJ function is ensured by the morphological specialization of pre-synaptic nerve terminals and post-synaptic AChR clusters. We further investigated the morphological changes during the onset of sarcopenia in the sarcopenic mice model. As shown in Fig. 5A, nerve terminals were distinct, branched and co-localized with endplate AChRs at 3 months old in the SAMP8. However, from 6 months onwards, nerve terminals became disorganized and terminal ends presented typical spherical shape; furthermore,
the area of endplate AChRs unoccupied by nerve terminals was increased. On the other hand, the control strain of SAMR1 showed no obvious changes of nerve terminals from 3 to 10 months. Until 12 months old, decreased nerve terminal branching and increased denervated AChRs area were observed.

Quantitative evaluation of NMJ morphology revealed that SAMP8 tended to have increased AChRs cluster fragmentation and discontinuity with increasing age, while SAMR1 mice presented no significant differences from 3 to 12 months old. At 10 and 12 months, SAMR1 showed significantly reduced cluster fragments and discontinuity compared with SAMP8 (fragmentation at 10 months and 12 months: \( p < 0.05 \) and \( < 0.01 \) respectively, discontinuity at 12 months: \( p < 0.05 \), Fig. 5B and C). Gradually increased cluster branching number from 8 to 12 months in SAMP8 could be taken as a compensatory mechanism to ensure the normal signal transduction at NMJ (Fig. 5D). In terms of AChRs cluster area and endplate area, they were found to be stable in the SAMR1 with increasing age, while they tended to be enlarged in the SAMP8 from 6 to 12 months of age. Compactness between AChRs cluster and endplate area was significantly reduced from 3 to 8 months of age (\( p < 0.01 \), Fig. 5E, F and G), indicating a process of AChRs cluster dispersion in sarcopenia.

The binding of Agrin and LRP4 can trigger the activation of MuSK, leading to AChRs clustering through Rapsyn. During this process, Dok7 plays an important role as a substrate for MuSK to maintain its activation. SAMP8 tended to have reduced Dok7 and Rapsyn protein expressions with ageing (Fig. 5H, K and L), explaining the morphological degeneration of endplate AChR clusters. The gradual increase in MuSK expression could also be taken as a compensatory mechanism for reduced Dok7 and Rapsyn (Fig. 5H and J). On the other hand, in SAMR1 without sarcopenic phenotype, MuSK tended to decrease during normal ageing and was expressed significantly lower than in SAMP8 at 10 months (\( p < 0.05 \), Fig. 5H and J). Both Dok7 and Rapsyn proteins tended to increase along with ageing in SAMR1 and were significantly overexpressed versus the SAMP8 at 10 months (\( p < 0.05 \) and \( < 0.05 \) respectively, Fig. 5H, K and L). Furthermore, SAMR1 showed larger Dok7 stained area aligned with AChRs cluster than SAMP8 at 10 months (Fig. 5I). Taken together, morphological degeneration of NMJ was found to happen at around 6 months old in SAMP8, preceding the occurrence of sarcopenia at 10 months of age. Besides, Dok7 was found to exhibit a difference between non-sarcopenic SAMR1 and sarcopenic SAMP8.

To further investigate and explain the mechanisms through which NMJ function was improved at month 6 post-LMHFV treatment, we also explored the effects of LMHFV on NMJ morphology. At month 4 post-treatment, cluster fragmentation and discontinuity were both alleviated (\( p < 0.01 \) and \( < 0.05 \) respectively, Fig. 6A, C and D). Increased AChRs cluster innervation and alleviated cluster discontinuity at month 6 post-treatment could contribute to the improved NMJ function (\( p < 0.05 \) and \( < 0.05 \) respectively, Fig. 6A, B and D). Although LMHFV presented no significant effects on cluster area and endplate area, the compactness between AChRs cluster area and endplate area were significantly increased at month 4 post-treatment (\( p < 0.05 \), Fig. 6A, E, F and G), indicating alleviated AChRs dispersion. Taken together, effects of the LMHFV treatment to the morphological degeneration include reduced AChE activity and elevated \( \alpha \) and \( \delta \) subunit expressions in the short term. Morphological degeneration of NMJ that was
mainly alleviated at month 4 post-treatment could be the reason that led to improved NMJ function at month 6 post-treatment in the sarcopenic SAMP8 mice.

**LMHFV promoted AChRs clustering and attenuated muscle atrophy by increasing Dok7 expression**

As LMHFV was observed to be effective in maintaining morphological integrity and thus the function of the NMJ during the onset of sarcopenia, we further investigate the molecular mechanism of its treatment effects. MuSK-Dok7-Rapsyn is the major pathway driving AChRs clustering. As shown in Fig. 7C, mRNA expression of Dok7 was significantly increased at month 4 post-treatment ($p < 0.05$). At the protein level, VIB group presented significantly increased Dok7 and Rapsyn expression at month 4 post-treatment ($p < 0.01$ and $< 0.05$ respectively) despite that Dok7 expression was reduced at month 2 post-treatment ($p < 0.05$, Fig. 7A and B). Furthermore, VIB group showed larger Dok7 stained area aligned with AChRs cluster at month 4 post-treatment (Fig. 7D), indicating Dok7 may be the key factor for the effects of LMHFV on NMJ.

To validate the role of Dok7 in vitro, primary myoblasts were isolated from SAMP8 mice. The time point of inducing differentiation was regarded as day 0 (D0) and with the co-staining of nucleus, myosin heavy chain (MHC) IIa and AChRs, mature myotubes were observed to be formed at D5 (Fig. 7E). LMHFV was treated in two different schemes: D1 to D6 or D5 to D10 (Fig. 7E) and it was found that LMHFV could significantly increase the formation of large AChRs cluster ($> 10 \mu m^2$) when applied from D1 to D6 but presented no effects on cluster formation when applied from D5 to D10 ($p < 0.001$, Fig. 7F and G), indicating LMHFV functioned at the myoblast differential phase in mediating AChRs cluster formation. In later in vitro experiments, LMHFV was applied from D1 to D6 only. Dok7 was then knocked down by shRNA transfection 12–16 hours before D0. Compared with sh-NC group, sh-NC + VIB group presented more AChRs cluster formation and larger myotube diameter ($p < 0.01$ and $< 0.01$ respectively, Fig. 7H, I and J). Knocking down Dok7 significantly suppressed AChRs cluster formation and reduced myotube diameter ($p < 0.05$ for both, Fig. 7H, I and J), which could not be retarded by LMHFV. LMHFV showed no significant effects on the mRNA expressions of Atrogin-1 and MuRF-1 in vitro (MuRF-1: $p = 0.058$, Fig. 7K), while sh-Dok7 group presented significantly increased MuRF-1 mRNA level ($p < 0.05$, Fig. 7K). At the protein level of MuSK-Dok7-Rapsyn pathway, LMHFV also significantly increased the protein expressions of Dok7 and Rapsyn in vitro ($p < 0.05$ and $< 0.05$ respectively, Fig. 7L, N and O). Knocking down Dok7 significantly increased MuSK expression but reduced Rapsyn level ($p < 0.01$ and $< 0.05$ respectively, Fig. 7L, M and O) and LMHFV could not retard the reduction of Rapsyn in myotubes with Dok7 knocked down (Fig. 7L and O). Dok7 protein expression in sh-Dok7 + VIB group was significantly higher than that in sh-Dok7 group, but still significantly lower than that in sh-NC + VIB group ($p < 0.05$ and $< 0.05$ respectively, Fig. 7L and N), which may be attributed to transfection efficiency. Taken together, these results indicated that LMHFV directly increased the expression of Dok7 at the NMJ that contributed to the observed improvements in AChRs clustering and attenuation of muscle atrophy.
LMHFV increased Dok7 expression through suppressing ERK1/2 phosphorylation

In skeletal muscles, mitogen-activated protein kinases (MAPKs) family is composed of three distinct signaling modules: extracellular signal-regulated kinase (ERK) 1/2, p38 and c-Jun N-terminal kinases (JNKs) \(^2\). MAPKs could transduce extracellular mechanical stress in an intensity-dependent manner and low-intensity or accustomed exercise mainly utilized ERK1/2 \(^2\)\(^4\). ERK1/2 is highly related to protein synthesis, mitochondrial function and obesity, so ERK1/2 functions as a common target in the nexus between sarcopenia and ageing \(^2\)\(^5\),\(^2\(^6\). Furthermore, activated ERK1/2 was reported to inhibit AChRs clustering \(^2\). Therefore, we hypothesized that LMHFV enhances Dok7 expression through the ERK1/2 or p38 mechanical signal transduction pathways. We observed that LMHFV significantly suppressed ERK1/2 phosphorylation at month 4 post-treatment but presented no significant effects on p38 phosphorylation (p < 0.001, Fig. 8A, B and C). Besides, LMHFV significantly reduced ERK1/2 phosphorylation in sarcopenic myoblasts \textit{in vitro} when applied from D1 to D6 of differentiation, yet no effects when applied from D5 to D10 (p < 0.01, Fig. 8D and E). These substantiated a previous study which reported that resistance exercise could reduce phosphorylation of ERK1/2 in skeletal muscles of old men \(^2\). After inhibition of ERK1/2 phosphorylation \textit{in vitro}, AChRs cluster formation was significantly increased (p < 0.001, Fig. 8F and G). As LMHFV could enlarge myotube diameter when applied from D1 to D6 and ERK1/2 was reported to function in inhibiting myoblast differentiation \(^2\), our results suggest that LMHFV promoted myoblast differentiation by suppressing ERK1/2 phosphorylation.

To further validate the relationship between ERK1/2 and Dok7, it was found that inhibiting ERK1/2 phosphorylation significantly increased the protein expressions of Dok7 and Rapsyn \textit{in vitro} (p < 0.01 and < 0.01, respectively, Fig. 8H and I), while knocking down Dok7 significantly suppressed ERK1/2 phosphorylation (p < 0.05, Fig. S4A and B). In myotubes with ERK1/2 phosphorylation inhibited, knocking down Dok7 still significantly reduced AChRs cluster formation and Dok7 protein expression (p < 0.05 and < 0.05 respectively), which could not be retarded by LMHFV (Fig. 8J, K, L and M). Therefore, LMHFV increased Dok7 expression through suppressing ERK1/2 phosphorylation.

Discussion

One of the pathological factors of sarcopenia is an apparent age-related decline in synaptic stability at the NMJ, manifesting as degenerative changes affecting both the pre-synaptic motor nerve terminal and the post-synaptic motor endplate \(^3\). Qualitative analyses of NMJs revealed conservation of synaptic structure across the entire lifespan in humans. Jones et al. \(^1\)\(^7\)’s study showed that qualitative analyses of NMJs suggested no signs of age-related degeneration or remodeling of synaptic structure across the entire lifespan in humans, yet our human study data showed that sarcopenia patients have distinct morphological and quantitative degeneration compared with non-sarcopenic patients.
In this study, NMJ degeneration in terms of both function and morphology was found to precede the onset of sarcopenia in SAMP8, whereas SAMR1 presented better NMJ function and more intact NMJ morphology than sarcopenic SAMP8 at 10 months old, indicating the possible causative relationship between NMJ degeneration and sarcopenia. SAMP8 presented reduced NMJ function from 6 to 8 months old, but no differences of NMJ function were observed in SAMP8 between 3–6 and between 8–12 months old, indicating NMJ functional degeneration in SAMP8 was abrupt and occurred rapidly. NMJ function is related to neurotransmitter release, AChE activity and endplate excitability. From 3 to 6 months in SAMP8, increased fragmentation and discreteness of AChRs cluster may lead to reduced endplate excitability while reduction in the size of nerve terminals may contribute to decreased release of ACh. To increase the binding between ACh and AChRs and ensure normal signal transduction at NMJ, AChE activity peaked at 6 months in SAMP8, which shortened the relative 1/2 RT. Furthermore, increased expressions AChR-α and -δ at 6 months promoted the binding between ACh and AChRs. As a result, no significant differences of NMJ function were observed between 3 and 6 months in SAMP8. From 6 to 8 months, the morphological degeneration of NMJ was not retarded in SAMP8 but both AChR-α and -δ expressions were reduced. As a compensatory mechanism, AChRs cluster area was expanded and accordingly, AChE activity was decreased to ensure sufficient ACh for binding with AChRs, resulting in increased relative 1/2 RT. However, increased ACh release would lead to the dispersion of AChRs (reduced compactness between AChRs cluster area and endplate area), which was also reported by other studies and this was related to a Cdk5-dependent mechanism. All these alterations would contribute to NMJ functional degeneration at 8 months in SAMP8. From 8 to 12 months, NMJ function was kept at a stable but low level in SAMP8. To ensure the normal signal transduction at NMJ, AChE activity was gradually reduced while AChR-α and -δ expressions tended to be increased. Besides, enlarged AChRs cluster area and increased cluster branching could adapt to increased fragments and discreteness of endplates. Furthermore, muscle atrophy was generally pronounced in SAMP8 from 8 and 12 months. SAMR1 mice showed larger muscle mass at 10 months than SAMP8, but there were no differences of AChRs cluster area compared with SAMP8, indicating muscle atrophy was always accompanied by expanded cluster and endplate area.

Our previous studies have reported that muscle strength and physical performance were significantly improved in the elderly receiving vibration treatment. In animal studies, we have also reported that LMHFV could significantly improve skeletal muscle structure and function in SAMP8 mice. In this study, grip strength was elevated at month 4 post-LMHFV treatment, which could be explained by increased tetanic force, although NMJ function was only improved at month 2 and 6 post-treatment. Mechanical vibrations applied to the muscles could elicit a reflex muscle contraction named ‘tonic vibration reflex’. Thus, the excitatory inflow during vibration stimulation was mainly related to the reflex activation of NMJ. LMHFV was not able to improve disrupted NMJ structure in short term but could enhance the sensitivity of the stretch reflex during vibration. LMHFV could further enlarge AChRs area, increase AChR-α and -δ expressions and reduce AChE activity at month 2 post-treatment. Numerous studies have suggested that cholinergic modulation and other functional consequences of AChE
inhibition may affect amyloid precursor protein processing and protect neurons against a variety of insults, while treatment of Alzheimer's disease has been dominated by the use of AchE inhibitors. Blotnick et al. reported in adult rats, horizontal treadmill training could elevate total AChE activity in fast muscles. In contrast, resistance exercise could significantly reduce AchE activity in rats with Alzheimer's disease. Hence, the effects of exercise on AChE activity were dependent on training types and physical conditions. At month 4 post-treatment, AChE activity in VIB group was still lower than that in CTL group, while AChR-α and-δ expressions were reduced, altogether leading to longer relative 1/2 RT. Furthermore, AChRs cluster fragmentation, discreteness and dispersion were attenuated at month 4 post-treatment. As a result, enhanced neurotransmission, retarded NMJ morphological degeneration and increased muscle force all contributed to the elevated grip strength of SAMP8 in VIB group at month 4 post-treatment. At month 6 post-treatment, denervation and AChRs cluster discreteness were alleviated, accompanied with relatively declined AchE activity, ultimately improving NMJ function.

Both of Dok7 and Rapsyn tended to be decreased with ageing, leading to ageing-related morphological degeneration of post-synapse in SAMP8. On the other hand, the protein levels of Dok7 and Rapsyn were generally increased in SAMR1 with increasing age and higher than those in SAMP8 at 10 months. This suggested in normal ageing process, increased Dok7 and Rapsyn could be considered as an adaption to the loss of muscle mass and strength, while decreased Dok7 and Rapsyn could accelerate the progression of sarcopenia. Aare et al.'s study also substantiated these results, which reported that old rats presented higher expression of Rapsyn than young rats, while sarcopenic mice (neurotrypsin over-expression) shower decreased level of Rapsyn compared with wild type. Activated MuSK could recruit Dok7 and lead to the anchoring of AChRs by Rapsyn. Increased MuSK could be considered as a compensatory mechanism for decreased Dok7 and Rapsyn in sarcopenic SAMP8. In contrast, during normal ageing, increased Dok7 and Rapsyn was able to antagonize the loss of muscle mass and strength and as its upstream factor, MuSK expression was relatively suppressed in SAMR1. Anagnostou et al. also reported that increased MuSK expression was a marker of denervation with ageing.

LMHFV could significantly increase the protein expressions of Dok7 and Rapsyn at month 4 post-treatment, which might lead to the improvements of AChRs cluster structure in terms of fragmentation and discreteness. Rapsyn was reported to be capable of inhibiting calpain activity, an enzyme involved in ACh-mediated AChRs cluster dispersal. This could also explain the attenuated dispersion of AChRs clustering at month 4 post-LMHFV treatment, although AChE activity was reduced. Hence, LMHFV could affect NMJ function in two distinct ways: reducing AChE activities and alleviating NMJ morphological degeneration by Dok7 and Rapsyn. Burden et al. reported Dok7, but not Rapsyn, was related to synapse-specific gene expression. Besides, only Dok7 mRNA expression was increased at month 4 post-treatment while in in vitro studies, knocking down Dok7 could reduce Rapsyn expression. So it was reasonable that LMHFV could increase the expression of Rapsyn by acting on Dok7. When Dok7 was knocked down in vitro, decreased myotube diameter and AChRs cluster formation validated the relationship among Dok7, AChRs clustering and sarcopenia. Besides, increased MuSK in myotubes
with Dok7 knocked down further confirmed that the increase of MuSK during ageing in SAMP8 could be taken as a compensatory mechanism \(^45\).

Our results showed that LMHFV suppressed ERK1/2 phosphorylation at month 4 post-treatment but presented no effects on the phosphorylation of p38. This was consistent with a previous study, revealing that the effects of exercise on MAPKs depended on training types, in which low intensity or accustomed exercise collectively utilized ERK1/2 \(^49\). Activated ERK1/2 was reported to inhibit myoblast differentiation \(^29\), explaining LMHFV could only suppress ERK1/2 phosphorylation and promote AChRs clustering when applied from D1 to D6 of differentiation. To further verify their relationship, blocking ERK1/2 phosphorylation was found to enhance AChRs clustering and increase Dok7 protein expression \textit{in vitro}. In myotubes with ERK1/2 phosphorylation blocked, knocking down Dok7 could still reduce AChRs cluster formation and Dok7 protein expression, which could not be retarded by LMHFV. Taken altogether, LMHFV should increase the Dok7 expression through suppressing ERK1/2 phosphorylation.

This study has a few limitations. Neuromuscular system in animal model may be different from in humans. Besides, the mice are quadrupedal while humans are bipedal, which they have different standing modes and loading patterns on the vibration platform. Also, although our study proved that LMHFV attenuated the progression of sarcopenia and NMJ degeneration by increasing Dok7 expression through suppressing ERK1/2 phosphorylation on the skeletal muscle end, other mechanical signal transduction mechanisms may also be involved on the neuron end.

In conclusion, we report an observed difference in NMJ morphology in sarcopenic hip fracture patients against non-sarcopenic patients. In sarcopenic SAMP8 model, functional and morphological degeneration of NMJ was found to precede the loss of muscle mass and performance. LMHFV attenuated NMJ degeneration and sarcopenia progression by increasing Dok7 expression for AChR clustering through suppressing ERK1/2 phosphorylation in skeletal muscle. Hence, targeting NMJ degeneration with mechanical stimulation is a promising biophysical intervention for the maintenance of neuromuscular health during the ageing process.

**Materials And Methods**

**Patients**

The research protocol for tissue sampling was approved by the Clinical Research Ethics Committee in the Chinese University of Hong Kong (Ref. 2021.008). Patients with hip fracture receiving hip arthroplasty were recruited and evaluated their sarcopenia status using AWGS 2019 definition and cutoffs \(^50\). Grip strength was used to assess muscle strength while ASMI was measured by DXA following our previous protocols \(^51\).

Briefly, subjects’ handgrip strength lower than the cut-off values of 18 kg and 26 kg for female and male, respectively and muscle quantity measured by DXA lower than the cut-off values of 5.4 and 7.0 kg/m\(^2\) for
female and male, respectively, would be defined as sarcopenia. The patient information was seen in Table S1.

**Animals**

Male SAMP8 (3, 6, 8, 10 and 12 months) and SAMR1 (3, 6, 8, 10 and 12 months) mice were obtained from the Laboratory Animal Service Center (LASEC), the Chinese University of Hong Kong, where male could avoid high hormonal variation. All animals were kept under conventional conditions, on a 12-hour light-12-hour dark cycle with food and water ad libitum. The research protocol was approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (Ref: 18/262/MIS).

**LMHFV treatment**

SAMP8 were applied with LMHFV treatment from 6 months old and followed up at three time points (month 2, 4, 6 post-treatment). SAMP8 in VIB group were housed individually in a standard and compartmented bottomless cage on the custom-designed vibration platform (V-Health Ltd, HKSAR, China). The LMHFV treatment was provided for 20 min/day and 5 days/week. The platform provided vertical cyclic oscillating vibration at 35Hz with a peak-to-peak acceleration of 0.3g (g = gravitational acceleration). All these settings were the same as our previous applications in clinical trials and animal studies.

For *in vitro* experiments, LMHFV was applied 20 min/day for 6 days *in vitro* (D1-D6 or D5-D10 of differentiation) with the culture plate directly on the vibration platform. The vibration platform provided vertical cyclic oscillating signal with settings same as *in vivo* study (35Hz, 0.3g).

**Grip strength measurement**

Handgrip strength of patients was measured by a dynamometer (5030JI, JAMAR, Bolingbrook, IL, USA) on the dominant hand. The maximum strength was taken from three attempts.

Grip strength of SAMP8 and SAMR1 was measured with a force gauge (Mark-10 Corporation, USA). Mice were held by the tail, while the mice grasped the grid connected to the force gauge with their fore paws. The tails of mice were pulled slowly until the mice released their fore paws from the grid and the peak force of each test was recorded.

**Skeletal muscle mass measurement in patients**

Total appendicular skeletal muscle mass (ASM) by DXA (Horizon, Hologic, Marlborough, MA, USA) was evaluated by segmented measurement of muscle mass at four limbs by operator-defined cutlines at specific anatomical landmarks. The ASM was then adjusted to the square of height to calculate the ASMI (expressed in kg/m²).

**Ex vivo skeletal muscle and NMJ functional test**

Details seen in Supplementary materials.
Histological and immunofluorescence analysis

Details seen in Supplementary materials.

Western blot analysis

Tibialis anterior (TA) muscles and cells for in vitro studies were harvested and digested for Western blot analysis according to our previous protocol. Primary antibodies used were: GAPDH (1:2000, MA5-15738, Invitrogen, USA), MuSK (1:2000, ab92950, Abcam, UK), Dok7 (1:2000, A9537, ABclonal, USA), Rapsyn (1:2000, A6716, ABclonal, USA), ERK1/2 (1:2000, 4695s, Cell Signaling Technology, USA), p-ERK1/2 (1:2000, 8544s, Cell Signaling Technology, USA), p38 (1:2000, 9212, Cell Signaling Technology, USA) and p-p38 (1:2000, 9216s, Cell Signaling Technology, USA). Secondary antibodies used were anti-rabbit IgG, HRP-linked antibody (1:5000, Cell Signaling Technology, USA) and goat anti-mouse IgG (H + L) antibody (1:5000, Invitrogen, USA). Relative protein contents were imaged by GeneGnome XRQ (Syngene, Cambridge, UK) and quantified by Image J software.

AChE activity assay

AChE activity was assessed with soluble proteins extracted from TA following manufacturer’s instruction (ab138871, Abcam, UK).

Gene expression analysis by real-time quantitative RT-PCR

Total RNA of EDL and cells was harvested for real-time quantitative RT-PCR following manufacturer’s instruction and our protocol. Gene-specific primers were included in Table. S2.

Isolation of primary myoblasts

Details seen in Supplementary materials.

Knocking down Dok7 and blocking ERK1/2 phosphorylation in vitro

The shRNA sequence against mouse Dok7 and scrambled negative control sequence were the same as previous studies. Plasmids encoding enhanced green fluorescent protein (EGFP) and Dok7 silencing/scrambled negative control sequences were obtained from Division of Life Science, The Hong Kong University of Science and Technology. Plasmids were transfected into myoblasts for 12–16 h before inducing differentiation by Lipofectamine 3000 Reagent (Thermo Scientific, Waltham, USA) based on the manufacturer’s instruction.

To block ERK1/2 phosphorylation in vitro, 1.5 µg/ml PD0325901 (Sigma-Aldrich, USA) was added at D0 of differentiation.

Statistics

All quantitative data were expressed as mean ± standard deviation. Significance was determined by either unpaired Student’s t tests or one-way analysis of variance (ANOVA) with post-hoc Bonferroni tests using
SPSS (SPSS Inc, IBM, USA). Significance was set at \( p < 0.05 \).

Declarations

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CONFLICT OF INTERESTS

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

ZB, SKHC, LQ, and WHC conceived and designed the project. WHC, LQ and SKHC obtained the funding. ZB and CC performed all experiments. CL helped to evaluate sarcopenia status of recruited patients. YL helped to perform Western blot. SKHC, CR, RMYW, LQ and WHC gave input to data analyses. RMYW collected clinical muscle biopsies. ZB, SKHC, WHC wrote the manuscript. WHC, SKHC, CR and LQ revised the manuscript. CR is an author of several issued and pending patents related to the use of low intensity vibration for the treatment of musculoskeletal injury and disease.

Supplementary information accompanies the manuscript on the Bone Research website http://www.nature.com/boneres.

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**Figures**

**Figure 1**

Morphological difference in NMJ is observed in sarcopenic and non-sarcopenic patients with hip fracture.
(A) Representative images of NMJ in sarcopenic and non-sarcopenic patients with hip fracture. (B, D and E) en face NMJ from 3 patients. AChRs cluster fragmentation, area and the compactness of AChRs cluster and endplate area presented no significant differences between sarcopenic and non-sarcopenic NMJ. (C) Sarcopenic NMJ presented more AChRs cluster number than non-sarcopenic NMJ (p<0.001).
Skeletal muscle degeneration in terms of muscle mass, function and gene expression in sarcopenic animal model (SAMP8) is suppressed by vibration treatment.

(A) n=6. Grip strength decreased significantly at 10 months in both SAMP8 and SAMR1 mice (SAMP8: p<0.001 compared with 3, 6 and 8 months old, SAMR1: p<0.05 and <0.01 respectively compared with 3 and 6 months old). At 10 months old, SAMR1 presented higher grip strength than SAMP8 (p<0.01).

(B) n=5-6. ex vivo triceps surae muscle tetanic force of SAMP8 mice arrived peak at 8 months compared with 3 months old, followed with a significant decrease at 10 months (p<0.05 and <0.01 respectively). SAMR1 mice showed no significant changes of tetanic force with ageing. At 10 months old, SAMR1 mice presented higher tetanic force than SAMP8 (p<0.05).

(C and D) n=5-6. The triceps surae muscle mass arrived peak at 8 months compared with 6 months but reduced significantly at 10 months old in SAMP8 (wet weight: p<0.05 and <0.01 respectively, CSA: p<0.05 for both). At 10 months old, SAMR1 presented larger triceps surae muscle mass compared with SAMP8 (wet weight: p<0.05, CSA: p<0.05). (E) n=4. At 10 months old, SAMP8 showed higher mRNA expressions of muscle atrophy related genes than SAMR1 mice (Atrogin-1: p<0.01, MuRF-1: p<0.05).

(F) Representative image of muscle fiber MHC staining in SAMP8 and SAMR1 mice at 10 months. (G) n=3. At 10 months, SAMR1 presented lower distribution of MHC I but higher composition of MHC IIb fibers than SAMP8 (p<0.001 and <0.05 respectively).

(H) n=6. LMHFV significantly elevated grip strength at month 4 post-treatment (p<0.05).

(I) n=5-6. At month 4 post-treatment, ex vivo tetanic force of triceps surae muscle was significantly increased in VIB group (p<0.01).

(J and K) n=6. LMHFV showed no significant effects on the triceps surae muscle mass.

(L) n=3-4. VIB group presented significantly reduced mRNA levels of Atrogin-1 and MuRF-1 at month 4 post-treatment (p<0.05 and <0.05 respectively).

(M and N) n=3. LMHFV significantly decreased MHC I fiber distribution but increased MHC IIb fiber composition at month 4 post-treatment (p<0.01 and <0.05 respectively).
Functional deterioration of the NMJ and related gene expressions observed in sarcopenic mice.

(A) n=5-6. At 10 months old, sarcopenic SAMP8 mice presented significantly increased neurotransmission failure than non-sarcopenic SAMR1. (B) n=5-6. Neurotransmission failure was significantly increased in SAMP8 mice at 8 months old compared with 6 months old. (C and D) n=5-6.
From 20th to 40th stimulus, compared with 6 months old, SAMP8 at 8 months old presented reduced intra-tetanic fatigue after direct muscle stimulations, but increased intra-tetanic fatigue after nerve stimulations. (E) n=5-6. Relative 1/2 RT between nerve and muscle stimulations in tetanic trains was significantly increased from 6 to 8 months in SAMP8 mice (p<0.01). (F) n=4. AChE activity was significantly increased from 3 to 6 months in SAMP8 mice, followed with a significant drop at 8 months (p<0.05 and <0.05 respectively). (G and H) n=3-4. In SAMP8 mice with increasing age, mRNA expressions of AChR-α and -δ subunit were significantly increased from 3 to 6 months, but presented significant decrease at 8 months old (α subunit: p<0.05 and <0.01 respectively, δ subunit: p<0.05 for both).

**Figure 4**

Vibration treatment alleviated NMJ functional deterioration associated with sarcopenia in sarcopenic mice

(A, B and C) n=5-6. LMHFV significantly reduced neurotransmission failure at month 2 and 6 post-treatment. (D, E and F) n=5-6. LMHFV showed no significant effects on the intra-tetanic fatigue of direct muscle stimulations. However, at month 2 and 6 post-treatment, VIB group presented significantly improved intra-tetanic fatigue after nerve stimulations at 40th and 50th stimulus (p<0.01 and <0.05 respectively). (G) n=5-6. Extended relative 1/2 RT in tetanic trainings was observed in VIB group at month
4 post-treatment (p<0.05). \((\text{H})\) \(n=4\). LMHFV significantly reduced AChE activity at month 2, 4 and 6 post-treatment (p<0.01, <0.05 and <0.05 respectively). \((\text{I})\) \(n=3\). LMHFV significantly increased AChR-\(\alpha\) and \(\delta\) subunit expressions at month 2 post-treatment (p<0.05 and <0.01 respectively), but mRNA levels of \(\alpha\) and \(\delta\) subunit were significantly reduced at month 4 post-treatment (p<0.05 and <0.05 respectively).

Figure 5
Morphological changes to NMJ during the onset of sarcopenia in sarcopenic mice.

(A) Representative images of NMJ in SAMP8 and SAMR1 mice from 3 to 12 months old. In SAMP8 mice, from 6 months old on, nerve terminals were disorganized, denervated AChRs area was increased and typical spherical ends of nerve terminals were observed. SAMR1 showed no obvious changes of nerve terminals from 3 to 10 months old. (B, C and D) n=41-51 en face NMJ from 5-6 mice. SAMP8 mice tended to have increased AChRs cluster fragmentation, discontinuity and branching with increasing age, while SAMR1 presented no significant changes during ageing. At 10 and 12 months, SAMR1 presented more intact NMJ structure than SAMP8 (fragmentation at 10 months and 12 months: p<0.05 and <0.01 respectively, discontinuity at 12 months: p<0.05). (E, F and G) n=41-51 en face NMJ from 5-6 mice. SAMP8 mice tended to present expanded AChRs cluster area and endplate area from 6 to 12 months old, but the compactness was significantly reduced at 8 months compared with 3 months old (p<0.01). AChRs cluster area and endplate area were stable in SAMR1 with increasing age. (H) Cropped western blots of MuSK, Dok7, Rapsyn and GAPDH in SAMP8 and SAMR1 mice from 3 to 12 months old. (I) Immunofluorescence images of AChRs and Dok7 in SAMP8 and SAMR1 mice at 10 months old. The distribution of Dok7 was aligned with AChRs cluster and SAMR1 showed larger Dok7 stained area than SAMP8. (J, K and L) n=4. SAMP8 tended to have reduced Dok7 and Rapsyn but increased MuSK protein expressions with ageing. In SAMR1 mice, MuSK tended to be reduced but Dok7 and Rapsyn were increased during ageing. At 10 months old, SAMR1 showed significantly higher expressions of Dok7 and Rapsyn but lower level of MuSK than SAMP8 (p<0.05, <0.05 and <0.05 respectively).
Figure 6

Vibration treatment alleviated NMJ morphological deterioration associated with sarcopenia in mice.

(A) Representative images of NMJ in SAMP8 of CTL and VIB group at month 2, 4 and 6 post-treatment. (B) n=11-21 en face NMJ from 5-6 mice. AChRs cluster innervation was significantly increased at month 6 post-treatment (p<0.05). (C and D) n=41-48 en face NMJ from 5-6 mice. At month 4 post-treatment, cluster fragmentation and discontinuity were significantly alleviated (p<0.01 and <0.05 respectively). Besides, LMHFV significantly decreased cluster discontinuity at month 6 post-treatment (p<0.05). (E, F and G) n=41-48 en face NMJ from 5-6 mice. LMHFV significantly increased the compactness between AChRs cluster area and endplate area at month 4 post-treatment (p<0.05), but cluster area and endplate area presented no significant differences.
Figure 7

LMHFV enhanced AChRs clustering during myotube and NMJ formation by increasing Dok7 expression.

(A) Cropped western blots of MuSK, Dok7, Rapsyn and GAPDH in SAMP8 mice of CTL and VIB groups. 
(B) n=3. VIB group showed significantly increased Dok7 and Rapsyn protein expressions at month 4 post-treatment (p<0.01 and <0.05 respectively), but Dok7 protein level was significantly reduced at month 2
post-treatment (p<0.05). (C) n=3-4. LMHFV significantly increased mRNA expression of Dok7 at month 4 post-treatment (p<0.05), but Dok7 mRNA level was significantly reduced at month 2 post-treatment (p<0.05). (D) Immunofluorescence images of AChRs and Dok7 in SAMP8 mice of CTL and VIB groups at month 4 post-treatment and VIB group presented larger Dok7 stained area than CTL group. (E) Mature myotubes were observed to be formed at D5 of differentiation with the co-staining of nucleus, MHC IIa and AChRs. (F and G) n=8-11 fields. LMHFV significantly promoted AChRs clustering (>10 μm²) when applied from D1 to D6 of differentiation but presented no effects on cluster formation when applied from D5 to D10 (p<0.001). (H and I) n=10-14 fields. Knocking down Dok7 significantly reduced AChRs cluster formation (p<0.05), which could not be retarded by LMHFV. (J) n=30-61 myotubes. sh-NC+VIB group presented larger myotube diameter (p<0.01). Knocking down Dok7 significantly reduced myotube diameter (p<0.05), which could not be retarded by LMHFV. (K) n=3. LMHFV showed no significant effects on the mRNA expressions of Atrogin-1 and MuRF-1 (p=0.058) in vitro. Knocking down Dok7 significantly increased MuRF-1 mRNA level (p<0.05). (L) Cropped western blots of MuSK, Dok7, Rapsyn and GAPDH in sh-NC, sh-NC+VIB, sh-Dok7 and sh-Dok7+VIB group in vitro. (M, N and O) n=3. LMHFV significantly increased the Dok7 and Rapsyn proteins in vitro (p<0.05 and <0.05 respectively). sh-Dok7 group showed significantly increased MuSK but reduced Rapsyn compared with sh-NC group (p<0.01 and <0.05 respectively). sh-Dok7+VIB group presented significantly higher Dok7 protein level than sh-Dok7 group, but significantly lower than that in sh-NC+VIB group (p<0.05 for both).
Figure 8

LMHFV increased expression of Dok7 by suppressing the activation of ERK1/2.

(A) Cropped western blots of p-ERK1/2, ERK1/2, p-p38, p38 and GAPDH in SAMP8 of CTL and VIB groups. (B and C) n=5. LMHFV significantly reduced ERK1/2 phosphorylation at month 4 post-treatment but presented no significant effects on p38 phosphorylation (p<0.001). (D) Cropped western blots of p-
ERK1/2, ERK1/2 and GAPDH in vitro. \textbf{(E)} n=3. LMHFV significantly suppressed ERK1/2 phosphorylation in sarcopenic myoblasts in vitro when applied from D1 to D6 of differentiation but showed no effects when applied from D5 to D10 (p<0.01). \textbf{(F and G)} n=10 fields. Blocking ERK1/2 phosphorylation significantly promoted AChRs cluster formation in vitro (p<0.001). \textbf{(H)} Cropped western blots of Dok7 and Rapsyn when ERK1/2 phosphorylation was blocked in vitro. \textbf{(I)} n=3. Blocking ERK1/2 phosphorylation significantly increased protein expressions of Dok7 and Rapsyn in vitro (p<0.01 and <0.01 respectively). \textbf{(J and K)} n=8-10 fields. In myotubes with ERK1/2 phosphorylation blocked, knocking down Dok7 significantly reduced AChRs cluster formation (p<0.05), which could not be retarded by LMHFV. \textbf{(L)} Cropped western blots of Dok7 in sh-NC, sh-Dok7 and sh-Dok7+VIB groups with all ERK1/2 phosphorylation blocked. \textbf{(M)} n=3. In myotubes with ERK1/2 phosphorylation blocked, knocking down Dok7 significantly reduced Dok7 protein expression (p<0.05), which could not be retarded by LMHFV.

\textbf{Supplementary Files}

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