Human Molecular Genetics, 2024, Vol. 33, 11, 935–944
https://doi.org/10.1093/hmg/ddae022
Advance access publication date 21 February 2024
Original Article

Vcp overexpression and leucine supplementation extend lifespan and ameliorate neuromuscular junction phenotypes of a SOD1^{G93A}-ALS mouse model

Tzyy-Nan Huang[1,†], Yu-Tzu Shih[1,2,†], Tzu-Li Yen[1], Yi-Ping Hsueh[1,*]

[1]Institute of Molecular Biology, Academia Sinica, 128 Sec 2, Academia Rd, Taipei, 11529, Taiwan, ROC
[2]Present address: Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA, USA.

*Corresponding author. Institute of Molecular Biology, Academia Sinica, 128 Sec 2, Academia Rd, Taipei 11529, Taiwan, ROC. E-mail: yph@gate.sinica.edu.tw
†Tzyy-Nan Huang and Yu-Tzu Shih contributed equally.

Abstract

Many genes with distinct molecular functions have been linked to genetically heterogeneous amyotrophic lateral sclerosis (ALS), including *SuperOxide Dismutase 1* (*SOD1*) and *Valosin-Containing Protein* (*VCP*). SOD1 converts superoxide to oxygen and hydrogen peroxide. VCP acts as a chaperon to regulate protein degradation and synthesis and various other cellular responses. Although the functions of these two genes differ, in the current report we show that overexpression of wild-type VCP in mice enhances lifespan and maintains the size of neuromuscular junctions (NMJs) of both male and female *SOD1*^{G93A} mice, a well-known ALS mouse model. Although VCP exerts multiple functions, its regulation of ER formation and consequent protein synthesis has been shown to play the most important role in controlling dendritic spine formation and social and memory behaviors. Given that *SOD1* mutation results in protein accumulation and aggregation, it may direct VCP to the protein degradation pathway, thereby impairing protein synthesis. Since we previously showed that the protein synthesis defects caused by *Vcp* deficiency can be improved by leucine supplementation, to confirm the role of the VCP-protein synthesis pathway in SOD1-linked ALS, we applied leucine supplementation to *SOD1*^{G93A} mice and, similar to *Vcp* overexpression, we found that it extends *SOD1*^{G93A} mouse lifespan. In addition, the phenotypes of reduced muscle strength and fewer NMJs of *SOD1*^{G93A} mice are also improved by leucine supplementation. These results support the existence of crosstalk between SOD1 and VCP and suggest a critical role for protein synthesis in ASL. Our study also implies a potential therapeutic treatment for ALS.

Keywords: amyotrophic lateral sclerosis; SuperOxide Dismutase 1; Valosin-Containing Protein; leucine

Introduction

Amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disorder, affects both upper and lower motor neurons, leading to muscle weakness and inability to move and breathe. The progressive motor neuron degeneration consequently results in patient death within 2 to 5 years after disease onset (https://medlineplus.gov/genetics/condition/amyotrophic-lateral-sclerosis/#causes) [1–3]. In the past three decades, over 60 genes have been linked to ALS (https://www.als.org/research/als-research-topics/genetics). Based on the molecular functions of ALS-linked genes, diverse pathogenic mechanisms have been associated with ALS—including protein aggregation, oxidative stress, gliosis, glutamate excitotoxicity, mitochondrial dysfunction, axonal transportation defect, ER stress, RNA metabolism, and proteasomal and autophagy impairment [2, 4–10]—highlighting the heterogeneity of ALS [11–14]. Moreover, in addition to the variety of ALS-linked genes, environmental factors also impact ALS phenotypes [14–16]. Therefore, a complex interplay between genes and environmental factors likely contributes to ALS [17].

The first causative gene of ALS to be identified was *SuperOxide Dismutase 1* (*SOD1*) [18], which accounts for 10%–20% of familial cases [19, 20]. The SOD1 protein is involved in converting superoxide radicals to oxygen and hydrogen peroxide [21, 22], indicating a role of oxidative stress in ALS [23, 24]. In addition, ALS-linked mutations result in aggregation and accumulations of SOD1 proteins in neurons [25–28]. Thus, similar to other neurodegenerative diseases caused by aberrant aggregation of misfolded proteins, proteinopathy is also a likely contributory factor to ALS [29, 30].

Valosin-containing protein (*VCP*) is another ALS-causative gene identified by exome sequencing of patients [31]. *VCP* encodes an AAA (ATPase Associated with diverse cellular Activities) ATPase protein that acts as a chaperon to control versatile processes in various types of cells, including endoplasmic reticulum-associated protein degradation (ERAD), the ubiquitin-proteasome system (UPS), autophagy, membrane fusion organelle formation of ER and Golgi apparatus, chromosome remodeling and transcriptional regulation [32–35]. In neurons, *VCP* knockdown or expression of disease-linked mutations of VCP reduces the density of dendritic spines, the subcellular structure of excitatory synapses [36–39], accounting for the nervous system dysfunction displayed by ALS patients. Although VCP exerts multiple functions, a previous study demonstrated that ER formation and

Received: November 23, 2023. **Revised:** January 12, 2024. **Accepted:** February 1, 2024

consequent protein synthesis are the most critical downstream activities of VCP in controlling the dendritic spine density of neurons [37]. Leucine supplementation that increases protein synthesis by promoting the mTOR pathway rescues dendritic spine density and the social behavioral deficits of *Vcp* mutant mice, further supporting a crucial role for the protein synthesis pathway in maintaining neuronal functions [37]. Consistent with that conclusion, increased protein intake improves the deficits of dendritic spines and social behaviors of *Vcp* mutant mice [39]. Thus, the ER-protein synthesis pathway controlled by VCP is crucial for dendritic spine formation and brain function.

Different VCP cofactors compete with each other for VCP binding and they target VCP to different processes [35, 40]. When a particular VCP-mediated cellular process predominates, VCP proteins are guided to and concentrated in that particular process. Consequently, other VCP-mediated processes do not function properly [37]. Accordingly, overexpression of wild-type (WT) VCP proteins may exhibit beneficial effects by minimizing the competition among different VCP cofactors for VCP binding, thereby meeting the demands of various VCP-mediated cellular processes [37, 41]. We have previously generated transgenic mice expressing WT *Vcp* and shown that these *Vcp* transgenic mice are generally healthy and fertile and display no alterations in brain anatomy or gross appearance [41]. Those *Vcp* transgenic mice serve as good models to investigate the possibility of crosstalk among various VCP downstream pathways.

Given that SOD1 mutation results in protein aggregation and accumulation [25–28], it may direct VCP to the protein degradation pathway and consequently minimize the contribution of VCP to other cellular processes due to insufficient VCP proteins for other pathways (Fig. 1). Under this scenario, impairment of the protein synthesis regulated by VCP may indirectly contribute to SOD1-linked ALS (SOD1-ALS). This hypothesis is consistent with a recent finding showing that VCP homeostasis is disrupted by SOD1 mutation in human induced pluripotent stem cells [42]. If our hypothesis is correct, we assume that *Vcp* overexpression is likely to ameliorate the phenotype of *SOD1* mutant mice. In this report, we crossed $SOD1^{G93A}$ mice, one of the most studied ALS mouse models [2, 13, 43], with two lines of WT *Vcp* transgenic mice, *i.e.* Vcp-H and Vcp-L [41]. Both Vcp-H and Vcp-L express a My-tagged WT *Vcp* transgene, with the ratio of Myc-tagged VCP to endogenous VCP being higher in Vcp-H (approximately 2:1) than in Vcp-L (approximately 1:1) [41]. We found that the $SOD1^{G93A}$ and *Vcp* double transgenic mice exhibit longer lifespans compared to $SOD1^{G93A}$ single transgenic mice. Consistent with this beneficial effect on lifespan, increased VCP protein levels elicited enhanced maintenance of NMJs in the $SOD1^{G93A}$ mice. To confirm the involvement of protein synthesis, we performed leucine supplementation by providing extra leucine in drinking water to $SOD1^{G93A}$ mice as leucine supplementation has been shown previously to promote protein synthesis and improve synapse formation and brain function [37, 39, 41, 44]. Similar to our results on *Vcp* overexpression, we found that leucine supplementation increases lifespan and maintains more NMJs of $SOD1^{G93A}$ mice. Thus, our study supports that, in addition to protein accumulation/aggregation, protein synthesis is a convergence point for ALS etiology, at least for SOD1- and VCP-related ALS.

Results

Vcp overexpression results in longer lifespan of $SOD1^{G93A}$ mice

Given that ER formation and consequent protein synthesis efficiency are critical downstream processes of VCP in controlling dendritic spine formation and brain function [37–39, 41], we speculated that, in addition to resulting in protein accumulation/degradation, disease-associated SOD1 mutations also impair protein synthesis by directing VCP to the protein degradation pathway and consequently influencing neuronal function (Fig. 1A and B). Under this scenario, promoting protein synthesis by means of *Vcp* overexpression or leucine supplementation would ameliorate the phenotype caused by *SOD1* mutation (Fig. 1C and D). To test our hypothesis, we first crossed $SOD1^{G93A}$ mice with transgenic mice overexpressing Myc-tagged wild-type Vcp, *i.e.* Vcp-L and Vcp-H mice, to generate double transgenic mice. In addition to being expressed in the brain [41], the Myc-tagged wild-type VCP proteins were also expressed in the spinal cord and muscles (Supplementary Fig. S1A and B). Myc-tagged VCP proteins were also expressed well in $SOD1^{G93A}$;Vcp-H double transgenic mice (Supplementary Fig. S1C). Survival curves, body weights and total moving distances in an open field were then measured every week to characterize the mouse phenotypes of all six different genetic backgrounds, *i.e.* WT, $SOD1^{G93A}$, Vcp-H, Vcp-L, $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice.

Our previous study showed that *Vcp* overexpression does not elicit noticeable phenotypes in the WT mouse background [41]. Similarly, we observed that both Vcp-H and Vcp-L mice were indistinguishable from WT mice in terms of survival, body weight and locomotor activity during the entire experimental period, regardless of sex (Fig. 2A–2F). For $SOD1^{G93A}$ mice, median survival was 151 days for males and 160 for females (Fig. 2A and B, Supplementary Table S1), which are comparable values to a previous report [43]. Importantly, both $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice exhibited significantly longer lifespans compared to $SOD1^{G93A}$ mice. The median survival of male $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice was extended to 171 and 172 days, respectively (Fig. 2A and B, Supplementary Table S1). For female $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice, it extended to 176 and 174 days, respectively (Fig. 2A and B, Supplementary Table S1). In addition, statistical analysis using a log-rank test indicated that the survival curves of male and female $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice differed from those of male and female $SOD1^{G93A}$ mice, respectively (Fig. 2A and B, Supplementary Table S1). These results indicate that *Vcp* overexpression extends the lifespan of $SOD1^{G93A}$ mice.

Although *Vcp* overexpression exerted a beneficial effect on the lifespan of $SOD1^{G93A}$ mice, its effect on body weight was very limited (Fig. 2C and D). Nevertheless, we observed that the walking distance of $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice in an open field was slightly longer than that of $SOD1^{G93A}$ mice, though only the data on male mice at D135 reached statistical significance (Supplementary Table S1, $SOD1^{G93A}$;Vcp-H vs. $SOD1^{G93A}$, $P = 0.034$). Thus, *Vcp* overexpression extends the lifespan of $SOD1^{G93A}$ mice but has a limited effect on body weight and locomotor activity.

Vcp overexpression maintains the NMJs of $SOD1^{G93A}$ mice

To further characterize the effect of *Vcp* overexpression, we measured the size of NMJs because NMJ degeneration is a consequence of motor neuron degeneration, one of the key features displayed by ALS mouse models [45, 46]. We identified NMJ areas based on the enzymatic activity of choline acetyltransferase (ChAT) in the soleus, a flexor muscle, and in the tibialis anterior muscle, an extensor muscle, of hindlimbs (Fig. 3A and B). These muscles, especially the soleus, are vital in movement and in maintaining a standing posture. We found that $SOD1^{G93A}$ mice exhibited reduced NMJ areas in both the soleus and tibialis anterior muscles compared to WT littermates at D150 (Fig. 3A–D),

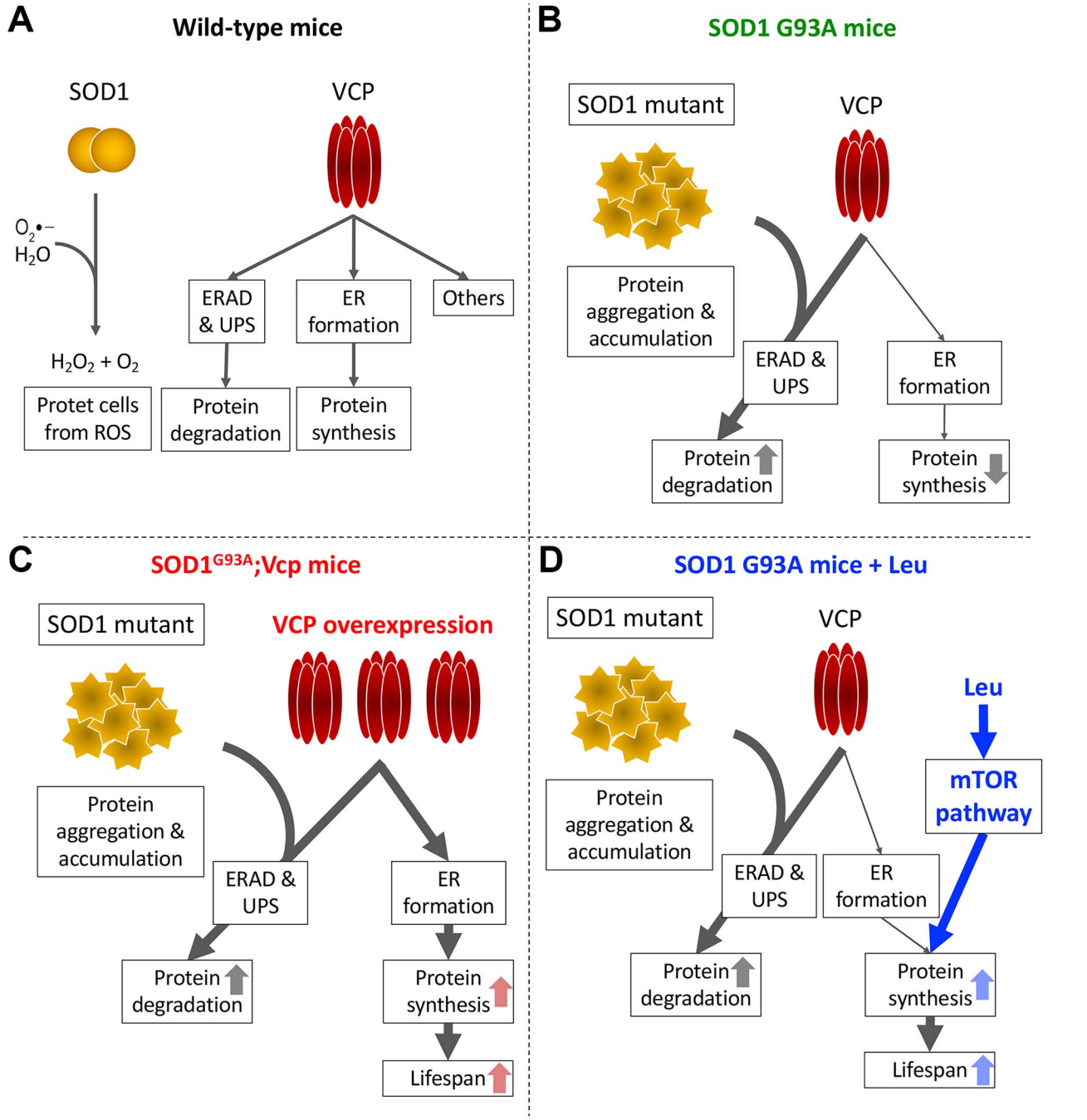

Figure 1. Models illustrating the crosstalk between SOD1 and VCP in ALS etiology. (A) The function of the SOD1 protein is to reduce oxidative stress by converting superoxide radicals to oxygen and hydrogen peroxide. VCP is a versatile chaperon controlling protein degradation (through ERAD, UPS and autophagy), protein synthesis (via regulation of ER formation) and other cellular processes (such as chromatin remodeling and transcriptional regulation). (B) In *SOD1*^{G93A} mice, the massive accumulation and aggregation of Sod1 mutant proteins and other proteins may direct Vcp to the protein degradation pathway, limiting the function of Vcp in protein synthesis and other activities. (C) When Vcp protein levels are increased, all demands for protein degradation and synthesis are met in *SOD1*^{G93A} mice. Thus, the lifespan of *SOD1*^{G93A} mice is increased, though the disease is not cured. (D) Independent from the Vcp-ER pathway, an increase in leucine amounts activates the mTOR pathway to promote protein synthesis, consequently ameliorating the phenotypes caused by *Sod1* G93A mutation. **Alt-text:** The massive accumulation and aggregation of SOD1 G93A mutant proteins and other proteins may direct VCP to the protein degradation pathway, limiting the function of VCP in protein synthesis and other activities. Thus, VCP overexpression and leucine supplementation that increases protein synthesis ameliorate the phenotypes caused by *SOD1* G93A mutation.

consistent with their impaired locomotion. Importantly, *Vcp* overexpression exerted a beneficial effect in terms of maintaining NMJs in *SOD1*^{G93A} mice because the NMJ areas of the soleus muscles of both *SOD1*^{G93A};Vcp-H and *SOD1*^{G93A};Vcp-L mice were larger than those of *SOD1*^{G93A} mice (Fig. 3A–D). For tibialis anterior muscles, NMJ areas were also enlarged in double transgenic mice, though only the data for *SOD1*^{G93A};Vcp-L mice reached statistical significance (Fig. 3D, *SOD1*^{G93A};Vcp-H vs. *SOD1*^{G93A}, $P = 0.032$).

Taken together, *Vcp* overexpression maintains more synaptic connections between motor neurons and muscles, which may account for the longer lifespan of *SOD1*^{G93A};Vcp-H and *SOD1*^{G93A};Vcp-L mice.

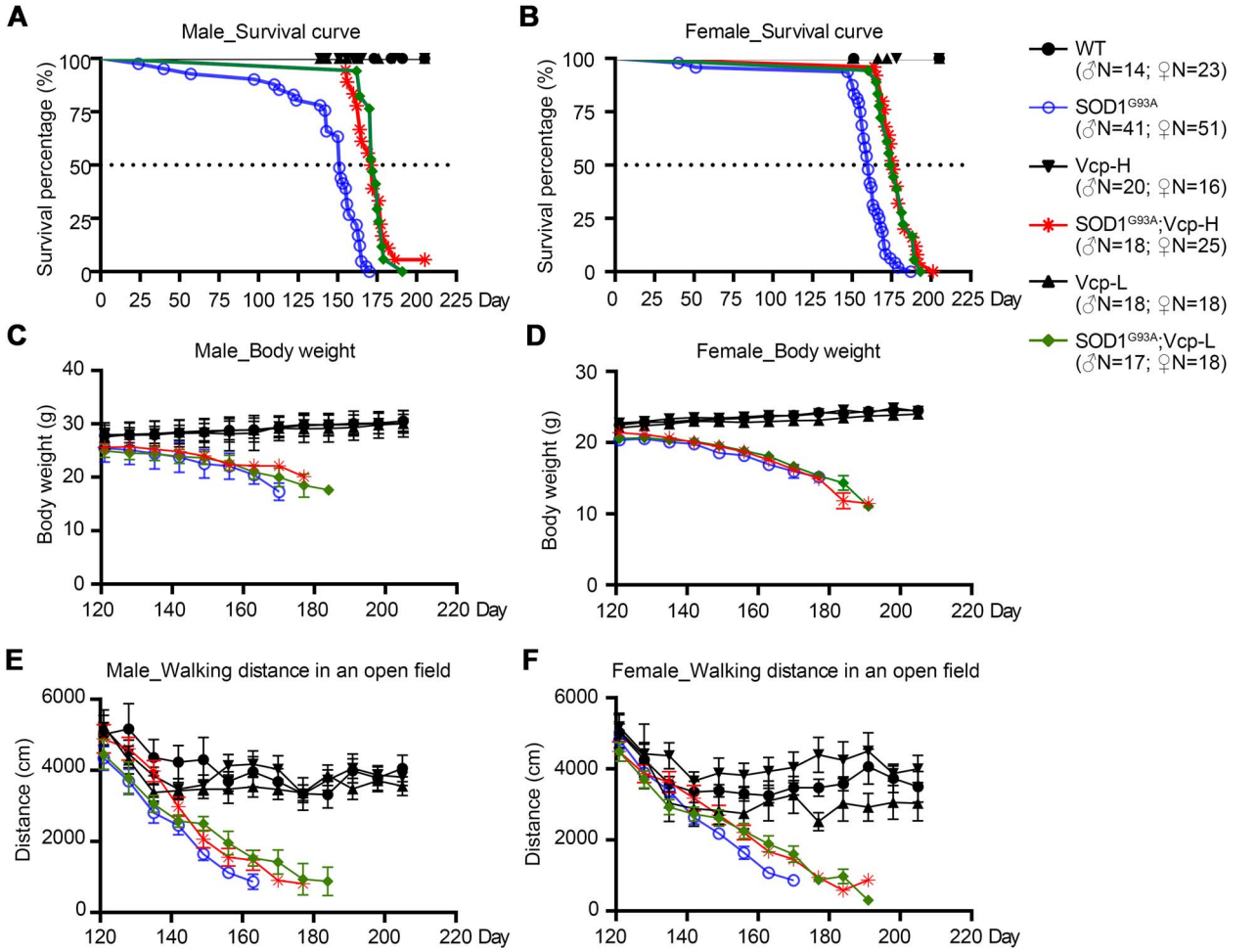

Figure 2. *Vcp* overexpression extends lifespan and slightly improves the locomotor activity of *SOD1*^{G93A} mice. Two *Vcp*-overexpressing transgenic mice, Vcp-H and Vcp-L, were crossed with *SOD1*^{G93A} mice to test the effect of *Vcp* overexpression on the ALS phenotypes of *SOD1*^{G93A} mice. Expression levels of the Myc-tagged *Vcp* transgene were approximately 2-fold higher in Vcp-H compared to Vcp-L [41]. The rescue effects of Vcp-H and Vcp-L were comparable with each other. The results of male and female mice are shown separately in the left and right panels, respectively. A total of six different mouse genotypes were compared. Given that mice died during experiments, only the sample sizes at the initial point are indicated in the legend. (A and B) Survival curves. Lifespans of *SOD1*^{G93A};Vcp-H and *SOD1*^{G93A};Vcp-L mice are longer than that of *SOD1*^{G93A} mice. (C and D) Body weight data. (E and F) The locomotor activity of mice was examined based on moving distance in an open field. Data represent mean ± SEM in (C)–(F). All statistical methods and results are summarized in Supplementary Table S1. **Alt-text:** Two *Vcp*-overexpressing transgenic mice, Vcp-H and Vcp-L, were crossed with *SOD1*^{G93A} mice and a total of six mouse lines were compared. The sample sizes at the initial point are indicated in the legend. (A and B) Survival curves. (C and D) Body weight. (E and F) The locomotor activity of mice was examined based on moving distance in an open field.

Leucine supplementation increases the lifespan, muscle strength and NMJ areas of *SOD1*^{G93A} mice

Given that protein synthesis is the critical downstream process of VCP in controlling neuronal functions, increased protein synthesis likely mediates the beneficial effect of *Vcp* overexpression on the lifespan of *SOD1*^{G93A} mice. To investigate that possibility, we subjected mice to leucine supplementation, which promotes protein synthesis via activation of the mTOR pathway. Extra leucine in drinking water was provided to *SOD1*^{G93A} mice and their WT littermates starting from D125. The median survival days of *SOD1*^{G93A} mice increased from D153 to D177 upon leucine supplementation (Fig. 4A, Supplementary Table S1), with a log-rank test supporting the statistically significant effect of leucine supplementation (Supplementary Table S1).

Similar to our results on *Vcp* overexpression, we found that leucine supplementation did not alter the body weight of mice (Fig. 4B, Supplementary Table S1). However, the muscle strength of *SOD1*^{G93A} mice with leucine supplementation did increase at D138, D145, D152 and D159 compared to *SOD1*^{G93A} mice that drank regular water (Fig. 4C, Supplementary Table S1; D138, $P = 0.032$; D145, $P = 0.003$; D152, $P < 0.001$; D159, $P = 0.003$). Consistent with this increase in muscle strength, the total NMJ area of *SOD1*^{G93A} mice significantly increased after leucine supplementation (Fig. 5A and B, Supplementary Table S1; soleus, $P < 0.001$; tibialis anterior, $P < 0.001$).

Taken together, our experiments reveal that an increase in leucine intake ameliorates the phenotype of *SOD1*^{G93A} mice. Moreover, the beneficial effect of leucine supplementation on *SOD1*^{G93A} mice is even more promising than that of *Vcp* overexpression.

Discussion

In the current report, we reveal crosstalk between two ALS-causative genes *SOD1* and *VCP*. *Vcp* overexpression and leucine supplementation extended the lifespan and ameliorated the muscle phenotypes of *SOD1*^{G93A} mice, echoing a previous study showing that the ER-protein synthesis pathway is the most critical downstream process of Vcp in controlling dendritic spine

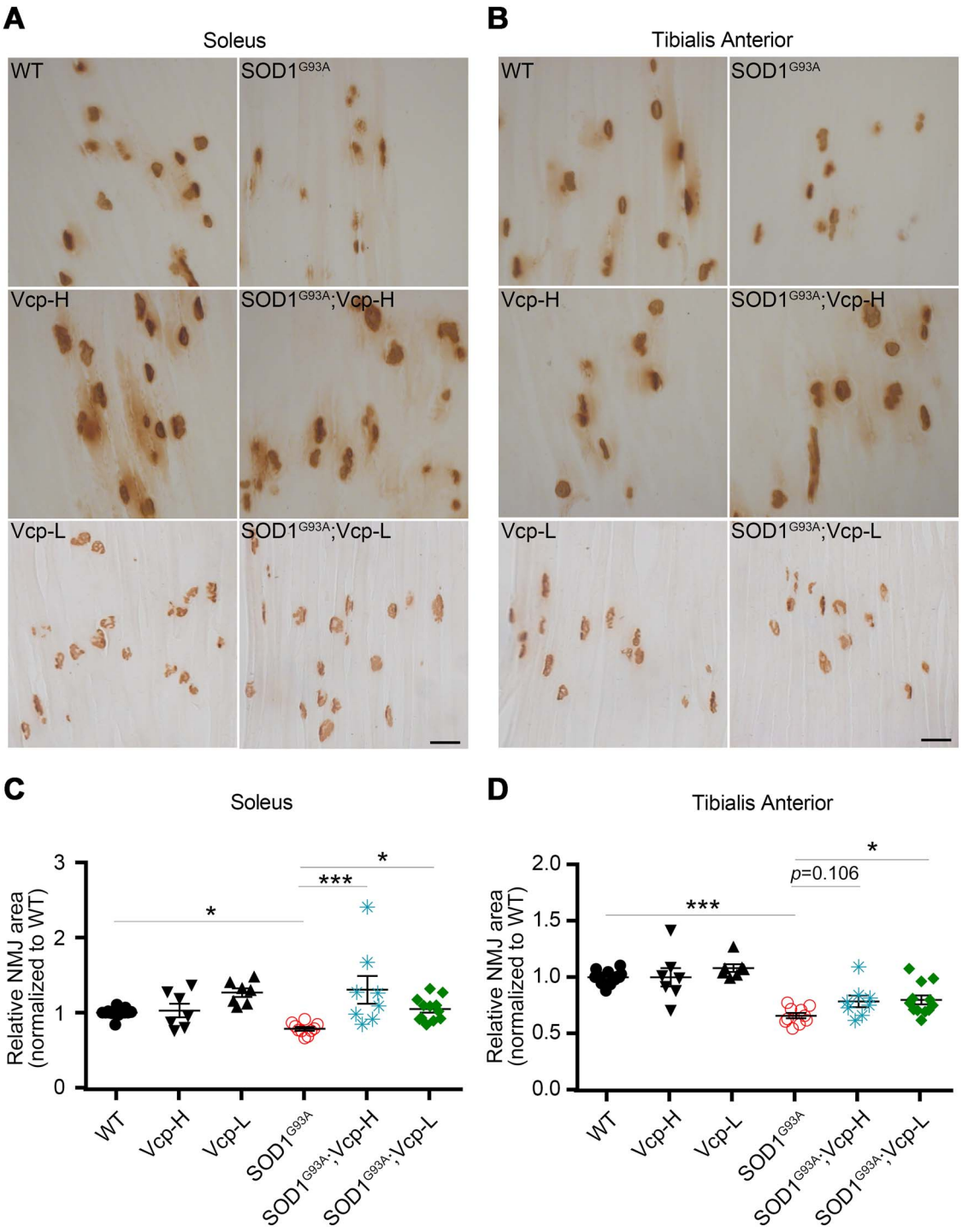

Figure 3. *Vcp* overexpression ameliorates the NMJ phenotypes of *SOD1*^{G93A} mice. The NMJs of all six different groups of mice listed in the panel were analyzed based on choline acetyltransferase (ChAT) activity at D150 before death. The results for male and female mice were pooled for analysis. The results for the soleus and tibialis anterior muscles of the hindlimbs are shown. (A and B) Representative images of ChAT activity. Scale bar: 100 μm. (C and D) Relative size of NMJs. All statistical methods and results are summarized in Supplementary Table S1. Sample size (N) of each group: WT, N = 13; Vcp-H, N = 7; Vcp-L, N = 7; SOD1^{G93A}, N = 10; SOD1^{G93A}; Vcp-H, N = 8; SOD1^{G93A}; Vcp-L, N = 12. Data represent mean ± SEM. The data points of individual mice are also shown. *$P < 0.05$; ***$P < 0.001$. **Alt-text:** The NMJs of the soleus and tibialis anterior muscles of the hindlimbs were analyzed based on choline acetyltransferase (ChAT) activity at D150 before death. The results for male and female mice were pooled for analysis. (A and B) Representative images of ChAT activity. Scale bar: 100 μm. (C and D) Relative size of NMJs. Data represent mean ± SEM. The data points of individual mice are also shown.

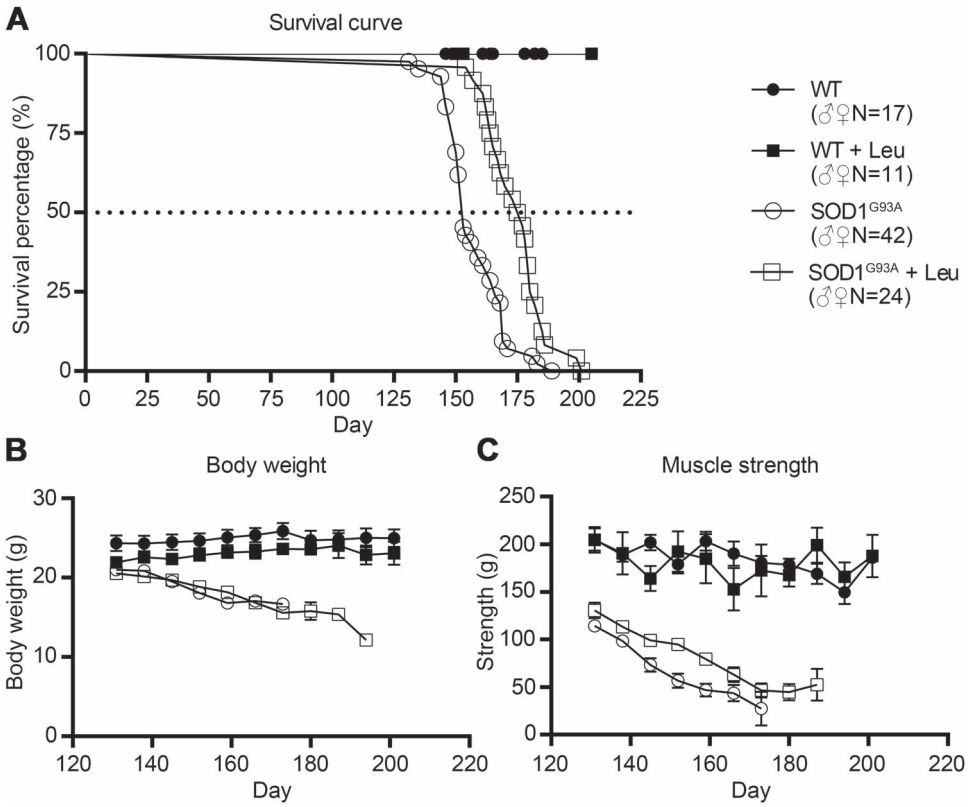

Figure 4. Leucine supplementation in drinking water started from D125 prolongs lifespan and increases muscle strength of *SOD1*^{G93A} mice. The results of male and female mice were pooled to analyze the effect of leucine supplementation. The sample sizes at the beginning of the experiments are shown. (A) Survival curves. The lifespan of *SOD1*^{G93A} mice subjected to leucine supplementation is longer than that of *SOD1*^{G93A} mice given regular drinking water. (B) Body weight data. (C) Muscle strength data. Data represent mean ± SEM in (B and C). All statistical methods and results are summarized in Supplementary Table S1. **Alt-text:** The results of male and female mice were pooled to analyze the effect of leucine (Leu) supplementation. The sample sizes at the beginning of the experiments are shown. (A) Survival curves. (B) Body weight data. (C) Muscle strength data. Data represent mean ± SEM in (B and C).

formation and mouse behaviors [37]. Thus, *SOD1* mutation may exert two negative effects on proteostasis; one is to increase protein aggregation/accumulation and the other likely reduces protein synthesis indirectly by directing VCP to protein degradation pathways (Fig. 1). Both *Vcp* overexpression and leucine supplementation increase protein synthesis in neurons [41], and these treatments may therefore ameliorate the phenotypes of *SOD1*^{G93A} mice. Thus, promoting protein synthesis by leucine supplementation represents a potential clinical treatment for ALS, although it cannot cure the disease.

In addition to the example of SOD1 and VCP shown in the current report, defective protein synthesis has been proposed recently to be relevant to the diverse defects caused by mutations of other ALS-causative genes [9]. The deficits elicited by mutations in *Fused in Sarcoma* (*FUS*), another gene associated with ALS, lead to a suppressed intra-axonal translation [47]. *FUS* mutation also reduces the translation of genes associated with mitochondrial function [48]. Moreover, through its RNA-binding activity, FUS has been shown to suppress the translation of Repeat Associated Non-AUG in a C9orf72-linked ALS model [49]. Thus, in addition to protein aggregation, defective protein synthesis is likely another point of convergence for ALS etiology. To explore this hypothesis further, it would be interesting in the future to investigate if *Vcp* overexpression or leucine supplementation also ameliorates the phenotypes caused by mutations in the *FUS* gene and other ALS-causative genes.

Branched-chain amino acids (BCAAs, including leucine, isoleucine and valine), are potent nutrient components that activate the mTOR pathway and enhance protein synthesis [50], with leucine exerting the most robust effect. Therefore, we selected leucine supplementation in the current study to test if it could counteract the deficits associated with *SOD1*^{G93A} mice. In our previous study, we showed that supplementation with leucine or a BCAA mixture increased protein synthesis and dendritic spine density, as well as improved social behaviors and memory performance, of *Vcp* mutant mice and other autism mouse models, including *Nf1* and *Cttnbp2* mutant mice [37, 39, 41, 44, 51]. Importantly, increased protein intake also elicits a similar effect in terms of improving the behavioral phenotype of *Vcp* mutant mice [39]. Therefore, it would be interesting to investigate in the future if an increase in total protein intake could ameliorate the phenotypes of SOD1-ALS and other ALS types.

The *VCP* gene has been linked to multiple neurological disorders, including Paget's disease of bone and frontotemporal dementia (IBMPFD) [52], frontotemporal dementia (FTD) and ALS-6 [31, 53], hereditary spastic paraplegia (HSP) [54], and autism spectrum disorders (ASD) [55]. To date, it remains unclear how *VCP* mutations elicit these different disorders. In the cases of IBMPFD, FTD and ALS, abnormal protein accumulations and aggregations are hallmarks of the diseases [52, 56, 57], which may impair protein synthesis indirectly and disrupt neuronal proteostasis by directing VCP and the related protein machinery to protein degradation pathways. Consequently, neuronal and brain functions are impaired. For HSP and ASD, the function of VCP in controlling ER formation and protein synthesis may be directly involved in regulating dendritic spine formation,

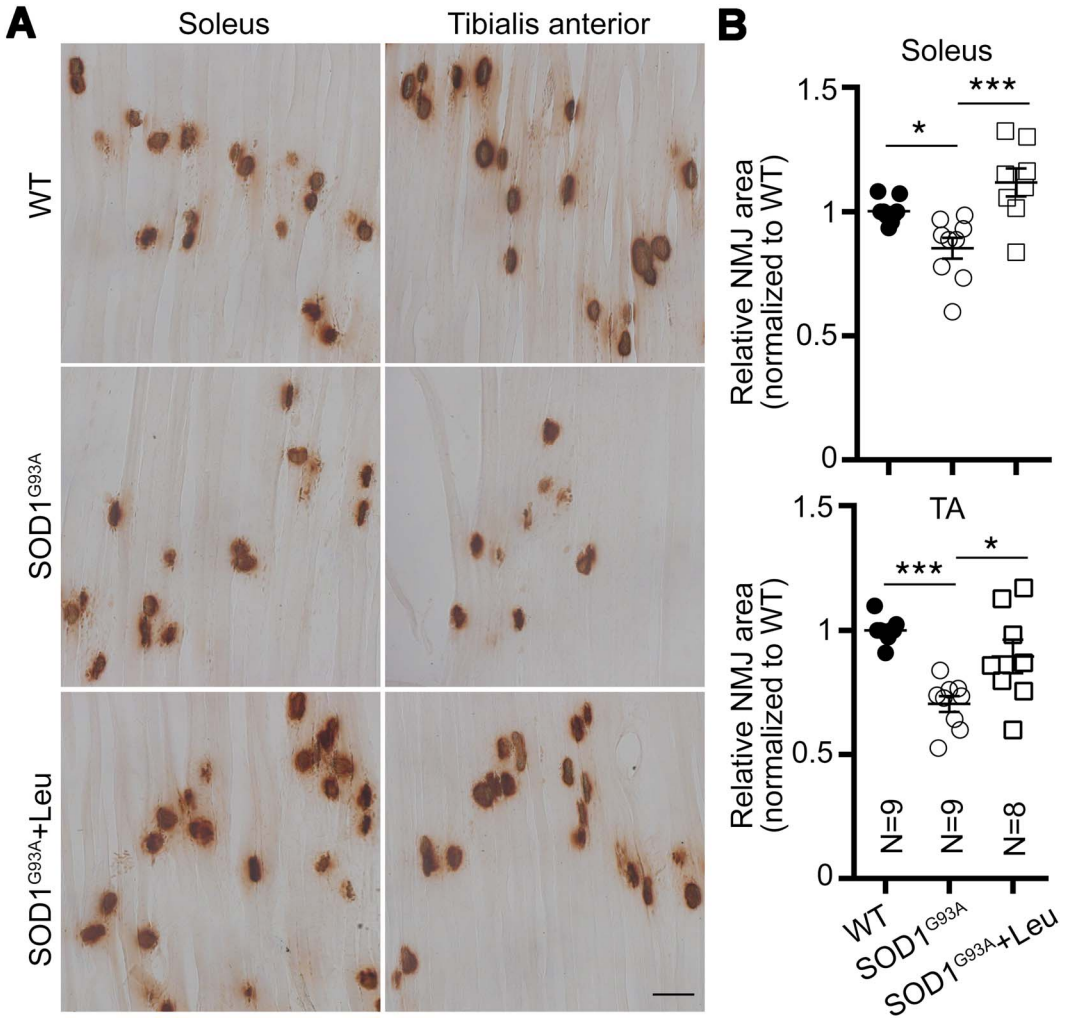

Figure 5. Leucine supplementation ameliorates NMJ phenotypes of *SOD1*^{G93A} mice. *SOD1*^{G93A} mice received leucine (Leu)-supplemented drinking water for 7 days starting at D125. The relative sizes of NMJ areas were then determined at D132. (A) Representative images of NMJs, as revealed by ChAT activity. Scale bar: 100 μm. (B) Relative size of NMJs. All statistical methods and results are summarized in Supplementary Table S1. Sample size (N) of each group: WT, N = 9; SOD1^{G93A}, N = 9; SOD1^{G93A} + Leucine, N = 8. Data represent mean ± SEM. The data points of individual mice are also shown. TA, tibialis anterior muscle. *$P < 0.05$; ***$P < 0.001$. **Alt-text:** *SOD1*^{G93A} mice received leucine (Leu)-supplemented drinking water for 7 days starting at D125. The relative sizes of NMJ areas were then determined at D132. (A) Representative images of NMJs, as revealed by ChAT activity. Scale bar: 100 μm. (B) Relative size of NMJs. Data represent mean ± SEM. The data points of individual mice are also shown. TA, tibialis anterior muscle.

neuronal function and behaviors [37–39, 41]. To further explore this possibility, the *Vcp*-overexpressing transgenic mice used in this report represent a useful tool for investigating the crosstalk between VCP and other disease-causative genes.

Materials and methods

Experimental design

The experiments in the current study are focused on exploring two topics. One was to examine the ameliorating effect of *Vcp* overexpression on *SOD1*^{G93A} mice. The other was to investigate the effect of leucine supplementation on *SOD1*^{G93A} mice. To minimize the phenotypic variation caused by different backcross generations, we compared mice of the same backcross generation. We performed *in vitro* fertilization to maximize the number of the same backcross generation. To test the effect of *Vcp* overexpression on *SOD1*^{G93A} mutation, *SOD1*^{G93A} mice were crossed with Vcp-H and Vcp-L, respectively, to generate *SOD1*^{G93A};Vcp-H and *SOD1*^{G93A};Vcp-L double transgenic mice. All littermates were used for analyses without selection bias. To test the effect of leucine supplementation, 1.8% L-leucine (LC Laboratories) in drinking water was freshly prepared daily and its provision began on day 125 (D125) and was maintained until the end of the experiment. Mice were randomly assigned to different groups. The effects of *Vcp* overexpression and leucine supplementation on NMJs were analyzed blindly. Body weight and locomotion or muscle strength were measured every week from D121. NMJs were analyzed at D132 or D150 before mice died.

Animals

All mice were housed in a 14 h:10 h light-dark colony room with *ad libitum* access to food (LabDiet 5010) and water. *SOD1*^{G93A} mice (stock No. 004435) [43] were imported from the Jackson Laboratory. *Vcp* transgenic mice, *i.e.* Vcp-H and Vcp-L, were generated and characterized previously [41]. *SOD1*^{G93A} male mice were crossed with Vcp-H and Vcp-L female mice, respectively, to generate *SOD1*^{G93A};Vcp-H and *SOD1*^{G93A};Vcp-L double transgenic mice. Note that "Vcp-L" and "6L" are interchangeable, likewise for "Vcp-H" and "21H". The L (low) and H (high) indicate the relative expression levels of Myc-VCP determined by immunoblotting [41].

The numbers 6 and 21 indicate the mouse line numbers. Since we kept only these two lines for study, we omitted the line numbers and used "H" or "L" to represent the mouse lines. Both male and female mice were subjected to analysis.

Ethics approval

Only mice were used in this study. All animal experiments were performed with the approval of the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 11-12-294) and in strict accordance with its guidelines and those of the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals.

Measurements of body weight, locomotion and muscle strength

Starting from D121, mice were subjected to body weight measurement every week. Meanwhile, locomotor activity (to test the effect of *Vcp* overexpression) or muscle strength (to investigate the effect of leucine supplementation) was analyzed immediately after body weight measurement.

Locomotion analysis in an open field

Mice were habituated in an experimental room for one hour before testing. After being individually placed into the central area of an open chamber (40 cm × 40 cm × 30 cm), mouse movement in the chamber was video recorded from above. The total travel distance of mice was quantified over 30 min using the Smart Video Tracking System (Panlab) [44, 51, 58].

Measurement of muscle strength

Mice were placed on a wire grid to keep their trunk in a horizontal position, with both forepaws and hindpaws touching the grid. We ensured that their forepaws held the wire grid before gently pulling mice backward by their tail. The maximal grip strength value was recorded using a grip strength instrument (FG_5005, Lutron). This procedure was repeated three times at 3-minute intervals, and the maximal measured value was selected to represent muscle strength.

NMJ analysis based on choline acetyltransferase (ChAT) activity

All WT, $SOD1^{G93A}$, Vcp-H, Vcp-L, $SOD1^{G93A}$;Vcp-H and $SOD1^{G93A}$;Vcp-L mice were subjected to cardiovascular perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS at D150. For the groups involved in leucine supplementation, mice were perfused for analysis at D132, *i.e.* one week after they began drinking leucine-supplemented water. The soleus and tibialis anterior muscles of hindlimbs were then collected to prepare cryosections using a Cryostat Microtome (CM 3050S, Leica). Half of the muscular sections (more than three sections) were then used for the ChAT activity assay. These sections were incubated in the incubating solution (0.1 M acetate buffer pH 5.2, 5 mM acetyl thiocholine iodide, 0.1 M sodium citrate, 30 mM copper sulfate in ddH_2O) for 6 h at room temperature (RT). After washing three times with PBS for a total of 30 min, the sections were incubated with 3% potassium ferricyanide for 10 min at RT to develop a brown precipitate. After extensively washing with PBS, the sections were dehydrated with 70% ethanol, 95% ethanol twice, and 100% ethanol twice, then coverslipped with Permount mounting medium. True-color images were then recorded using an Axioimager M2 microscope (Carl Zeiss). All areas of NMJ-positive signals on the sections were collected for quantification using ImageJ (NIH). Total NMJ areas were normalized to the WT of each group of experiments.

Statistical analysis

All quantitative data are shown as means plus/minus standard error of the mean (SEM) as indicated in each figure legend. Graphs were plotted using GraphPad Prism 7.0 (GraphPad software). The sample sizes of analyzed animals were gradually reduced because of the lethality caused by *SOD1* mutation. The sample sizes indicated in the figures are the numbers of mice at the initial points. Survival rates were analyzed using the log-rank test with Holm-Sidak's post-test. Other statistical analyses were performed using one-way analysis of variance (ANOVA, for leucine supplementation experiments) and two-way ANOVA (for *Vcp* overexpression experiments) with Bonferroni's post-test correction. All statistical analyses were performed using Sigmastat 3.5 (SigmaPlot software). P values < 0.05 were considered significant. All statistical results are summarized in Supplementary Table S1.

Supplementary data

Supplementary data is available at *HMG Journal* online.

Conflict of interest statement: The authors declare no conflict of interest.

Funding

We thank the Transgenic Core Facility (supported by Academia Sinica, AS-CFII-108-104) and the Animal Facility of the Institute of Molecular Biology, Academia Sinica, for excellent technical assistance. Dr John O'Brien conducted English editing. This work was supported by grants from Academia Sinica (AS-IA-106-L04 and AS-IA-111-L01) to Y.-P.H. Y.-T.S. was supported by a postdoctoral fellowship from Academia Sinica.

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