Amyloid precursor protein (APP) contributes to pathology in the SOD1<sup>G93A</sup> mouse model of amyotrophic lateral sclerosis

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In amyotrophic lateral sclerosis (ALS), the progressive loss of motor neurons is accompanied by extensive muscle denervation, resulting in paralysis and ultimately death. Upregulation of amyloid beta (A<sub>4</sub>) precursor protein (APP) in muscle fibres coincides with symptom onset in both sporadic ALS patients and the SOD1<sup>G93A</sup> mouse model of familial ALS. We have further characterized this response in SOD1<sup>G93A</sup> mice and also revealed elevated levels of β-amyloid (Aβ) peptides in the SOD1<sup>G93A</sup> spinal cord, which were predominantly localized within motor neurons and their surrounding glial cells. We therefore examined the effect of genetic ablation of APP on disease progression in SOD1<sup>G93A</sup> mice, which significantly improved multiple disease parameters, including innervation, motor function, muscle contractile characteristics, motor unit and motor neuron survival. These results therefore strongly suggest that APP actively contributes to SOD1<sup>G93A</sup>-mediated pathology. Together with observations from ALS cases, this study indicates that APP may contribute to human ALS pathology.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder in which progressive loss of upper and lower motor neurons, accompanied by neuromuscular junction (NMJ) denervation, leads to diminished connectivity between the motor circuitry within the central nervous system (CNS) and the peripheral skeletal musculature. The resulting decline in voluntary motor function culminates in paralysis and ultimately death, typically within 3–5 years of symptom onset (1). High-copy SOD1<sup>G93A</sup> transgenic mice are commonly used to model ALS and recapitulate much of the pathophysiology of the human disease, including progressive motor neuron degeneration, functional impairment and reduced life-span (2). Evidence from mutant SOD1 mouse models has revealed that non-neuronal cell types, including astrocytes (3,4), microglia (5), and Schwann cells (6), play important accessory roles in the selective degeneration of motor neurons.

Muscle fibres, the post-synaptic target of lower motor neurons, have also been postulated to contribute to motor neuron death in ALS (7). Recent evidence from transgenic mice in which ALS-linked mutant SOD1 expression is restricted to muscle fibres have shown that muscle is itself a primary target of mutant SOD1-mediated toxicity (8) and that this can trigger motor neuron degeneration (9). Additionally, muscle fibre-specific upregulation of the neurite growth inhibitor, Nogo-A, has been shown to occur in both SOD1<sup>G86R</sup> mice, as well as in human sALS cases (10). Moreover, genetic ablation of Nogo-A ameliorates disease progression in SOD1<sup>G86R</sup> mice, while ectopic overexpression of Nogo-A in wild-type (WT) mouse muscle results in motor axon terminal degeneration (11). These findings support the hypothesis that ALS may be a ‘dying-back’ disease,

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show that NMJ loss and motor neuron degeneration are sub-
peptides are predominantly localized within affected motor
with symptom onset in SOD1G93A medial gastrocnemius
stage (120 days) disease time points (Fig.1A–C) confirmed
symptomatic (50 days), late-symptomatic (85 days) and late-
Immunohistochemical analysis of muscle at early-
secondarily to denervation
Muscle fibres. Additionally, we show that A
b levels are elevated in
slow-twitch fibres. Furthermore, once triggered, this response
could exacerbate ongoing degeneration of motor axon termi-
als in SOD1G93A-mediated disease.

RESULTS
APP upregulation correlates with type-II muscle fibre
atrophy in symptomatic SOD1G93A mice and occurs
secondarily to denervation

Immunohistochemical analysis of muscle at early-
symptomatic (50 days), late-symptomatic (85 days) and late-
stage (120 days) disease time points (Fig. 1A–C) confirmed
that upregulation of APP manifests concomitantly with symptom onset in SOD1G93A medial gastrocnemius
(MGC) muscle fibres, as previously reported (18). APP
immunoreactivity was not observed in WT muscle (Fig. 1D)
or in 35-day SOD1G93A muscle (Supplementary Material, Fig. S1A). Although the number of APP-positive fibres and the
labelling intensity increased with disease progression, APP expression remains restricted to a subpopulation of
muscle fibres, even at late-stage disease. Quantification of
muscle fibre cross-sectional area (CSA) revealed a general
shift to a smaller average CSA in SOD1G93A versus WT
gastrocnemius (Fig. 1E). However, the reduced CSA was exa-
cerbated in APP-positive muscle fibres, indicating a correl-
ation between the APP response and muscle fibre atrophy.
To investigate the temporal relationship between APP upregu-
lation and muscle fibre denervation, the sciatic nerve was axo-
tomized in WT mice and the APP response was examined in
affected hind-limb muscles after 14 days. Immunohistochem-
istry revealed that upregulation of APP within muscle fibres is
a reactive response triggered by denervation (Fig. 1F). More-
over, despite the complete denervation of all muscle fibres
induced by axotomy, the APP response remained restricted to
a subpopulation of muscle fibres, the distribution of which resembled that observed in SOD1G93A mice.

The distribution of APP-positive muscle fibres in the super-
ficial gastrocnemius in SOD1G93A mice indicated that they are
likely to be type-IIB fibres, which are innervated by fast-firing
phasic motor neurons that are preferentially vulnerable to
SOD1G93A-mediated disease (13,14). Indeed, immunostaining
analysis of APP and class-specific myosin markers in serial
sections from late-stage SOD1G93A MGC indicated that APP
upregulation occurs exclusively in type-IIB fibres and was
not observed in type-IIa, -IIX or -IIC fibres (an example of
each fibre type is highlighted in Fig. 1G and H). Additionally,
the APP response was not observed in diaphragm muscle
(Fig 1I), which undergoes a 40% loss of innervating motor
axons by end-stage (23), but which lacks type-IIB fibres in mice [Supplementary Material, Fig. S1B and (24)]. This
further supports the specificity of the reactive APP response
to denervated type-IIb muscle fibres.

Rather than being a primary trigger of NMJ denervation,
APP upregulation appears to be a secondary effect of denerv-
ation in SOD1G93A-mediated disease. However, since overex-
ression of APP in muscle fibres is detrimental for NMJ
formation (19), it is possible that the APP response in SOD1G93A mouse muscle may inhibit compensatory
re-innervation of type-IIb fibres and thereby prioritize the rein-
nervation of more disease-resistant (APP-negative) slow-twitch fibres. Furthermore, once triggered, this response
could exacerbate ongoing degeneration of motor axon termi-
inals in SOD1G93A-mediated disease.

Soluble β-amyloid levels are elevated in spinal cord, but
not muscle, in SOD1G93A mice

To determine whether amyloidogenic processing of APP, fol-
lowing its upregulation in SOD1G93A mouse muscle, results in
increased β levels, levels of soluble βx-40 and βx-42 were quantified in gastrocnemius muscle tissue from WT and
SOD1G93A mice by enzyme-linked immunosorbent assay
(ELISA). However, in agreement with a previous report
(18), levels of soluble βx-40 (Supplementary Material, Fig. S2) in muscle tissue from both WT and SOD1G93A
response to type-IIb muscle fibres. Scale bars (in F–I) correspond to type-IIb muscle fibres in (H), whereas subtypes of other type-II fibres are negative for both markers (superimposed lower-case red); type-IIc (hybrid) muscle fibres appear positive for both markers and intensity of APP immunoreactivity increases by 85 days (G) as determined for WT (n = 3), as well as APPpos and APP-negative (APPneg) populations of muscle fibres from (84 days) SOD1G93A (n = 3) MGC muscle, CSA was significantly reduced in APPpos versus APPneg muscle fibres (P ≤ 0.005; determined by two-tailed t-test). (F) Sciatic nerve fragment section was immunohistochemically labelled for APP (G), while the adjacent section from the series was immunofluorescently labelled with two myosin isoform-specific monoclonal antibodies (H) that recognize type-Ib muscle fibres (clone BF-F3, shown in green) and type-IIa muscle fibres (clone SC-71, shown in red); type-Iic (hybrid) muscle fibres appear positive for both markers and type-Ix fibres are negative for both markers (superimposed lower-case letters denote an example of each fibre type); type-I fibres are absent in this region of the MGC (data not shown). All APP-positive muscle fibres in (G) corresponded to type-IIb muscle fibres in (H), whereas subtypes of other type-II fibres all appeared negative for APP upregulation. Negative APP labelling in diaphragm muscle, which does not contain type-IIb muscle fibres, is evident in all ventral horn neurons in 120-day SOD1G93A mice. Moreover, the restricted localization of these potentially toxic peptides suggests that they likely achieve significantly higher local concentrations than that suggested by the ELISA analysis of whole lumbar spinal cord homogenates and that this may contribute to pathology in SOD1G93A-mediated disease. A similar staining pattern was observed for an antibody raised against the rodent-specific N-terminus of the Aβ peptide (Aβ1–16; Supplementary Material, Fig. S3), thus indicating that the full-length Aβ42 peptide and not the non-toxic α-secretase product accounted for the immunoreactivity described here. These results are in keeping with our quantitative ELISA data that show enhanced levels of Aβ40 and Aβ42 in 120-day SOD1G93A mice. Moreover, the restricted localization of these potentially toxic peptides suggests that they likely achieve significantly higher local concentrations than that suggested by the ELISA analysis of whole lumbar spinal cord homogenates and that this may contribute to pathology in SOD1G93A-mediated disease.

Figure 1. APP upregulation in type-II muscle fibres correlates with atrophy and occurs secondarily to denervation in SOD1G93A muscle. APP immunoreactivity is not observed in WT MGC muscle fibres (A) but becomes evident in a subpopulation of superficial MGC fibres in SOD1G93A mice at 50 days (B). In SOD1 mice, the abundance of APP-positive (APPpos) fibres and intensity of APP immunoreactivity increases by 85 days (C), while atrophy of the APPpos population of MGC muscle fibres is pronounced by 120 days (D) (scale bar = 200 μm). Average muscle fibre diameter (E) was determined for WT (n = 3), as well as APPpos and APP-negative (APPneg) populations of muscle fibres from (84 days) SOD1G93A (n = 3) MGC muscle. CSA was significantly reduced in APPpos versus APPneg muscle fibres (P ≤ 0.005; determined by two-tailed t-test). (F) Sciatic nerve fragment was carried out in 21-day-old WT mice and 14-day later MGC muscles were examined for APP immunoreactivity which exhibited a similar pattern to that of symptomatic SOD1G93A MGC muscle. A transverse section from symptomatic (100 days) SOD1G93A MGC muscle was immunohistochemically labelled for APP (G), while the adjacent section from the series was immunofluorescently labelled with two myosin isoform-specific monoclonal antibodies (H) that recognize type-Ib muscle fibres (clone BF-F3, shown in green) and type-IIa muscle fibres (clone SC-71, shown in red); type-Iic (hybrid) muscle fibres appear positive for both markers and type-Ix fibres are negative for both markers (superimposed lower-case letters denote an example of each fibre type); type-I fibres are absent in this region of the MGC (data not shown). All APP-positive muscle fibres in (G) corresponded to type-IIb muscle fibres in (H), whereas subtypes of other type-II fibres all appeared negative for APP upregulation. Negative APP labelling in diaphragm muscle, which does not contain type-IIb muscle fibres, is evident in all ventral horn neurons in 120-day SOD1G93A spinal cord, however, as with the APP labelling, a population of non-neuronal (β-III tubulin negative) cells in the ventral parenchyma were also immunoreactive for Aβ42 (Fig. 2D, lower panels). As suggested by the morphology of these non-neuronal cells, double-labelling with the astrocytic marker, glial fibrillary acidic protein (GFAP), confirmed that these cells were indeed astrocytes (Fig. 2E, lower panels). The elevation of Aβ42 in astrocytes at late-stage disease in SOD1G93A spinal cord may reflect either enhanced amyloidogenic processing of APP in astrocytes, possibly due to oxidative stress, or may be due to endocytosis of Aβ42 species released from neurons. Although astrocytes were present in WT spinal cord, Aβ42 immunoreactivity was not colocalized with GFAP labelling (Fig. 2E, upper panels). A similar staining pattern was observed for an antibody raised against the rodent-specific N-terminus of the Aβ peptide (Aβ1–16; Supplementary Material, Fig. S3), thus indicating that the full-length Aβ42 peptide and not the non-toxic α-secretase product accounted for the immunoreactivity described here. These results are in keeping with our quantitative ELISA data that show enhanced levels of Aβ40 and Aβ42 in 120-day SOD1G93A mice. Moreover, the restricted localization of these potentially toxic peptides suggests that they likely achieve significantly higher local concentrations than that suggested by the ELISA analysis of whole lumbar spinal cord homogenates and that this may contribute to pathology in SOD1G93A-mediated disease.
Together with the correlation between APP upregulation and hallmarks of muscle fibre atrophy, these data indicate that APP could potentially contribute to SOD1G93A-mediated motor neuron degeneration via multiple mechanisms. Therefore, in order to establish whether APP does actively contribute to ALS-like pathology, the effect of removing APP on disease progression in SOD1G93A mice was investigated.

Generation of APP-knockout SOD1G93A cross mice

APP-knockout [APP<sup>−/−</sup>, (25)] and SOD1G93A mice were crossbred to generate progeny of the following genotypes: APP<sup>+/+</sup> (i.e. WT), APP<sup>−/−</sup>, APP<sup>−/+</sup>, APP<sup>−/−</sup>:SOD1G93A (i.e. SOD1<sup>G93A</sup>, APP<sup>−/−</sup>:SOD1<sup>G93A</sup> and APP<sup>−/−</sup>:SOD1<sup>G93A</sup>). The absence of APP protein in APP<sup>−/−</sup>:SOD1<sup>G93A</sup> mouse tissue was confirmed by immunoblotting (Supplementary Material, Fig. S4A). SOD1<sup>G93A</sup> protein expression (Supplementary Material, Fig. S4A and B) and copy number (Supplementary Material, Fig. S4C) were not affected by genetic ablation of APP.

APP ablation dramatically improves NMJ innervation in SOD1G93A mice

In view of the specificity of the APP response to vulnerable muscle fibres and its possible deleterious effects on innervation, we first examined the effect of APP ablation on NMJ innervation in late-stage (120-day) SOD1<sup>G93A</sup> mice by assessing colocalization of fluorescently labelled pre-synaptic and post-synaptic NMJ markers in the extensor digitorum longus (EDL) muscle. This muscle was selected since, like the MGC, it is primarily composed of type-IIb fibres but it is small enough to quantify all NMJs, whereas the large size of the MGC would necessitate sampling.
which could introduce an undesirable variable. NMJs were classed as ‘innervated’, ‘partially innervated’ or ‘denervated’ (an example of each category is shown in Fig. 3A).

As expected, by late-stage disease, a large proportion of NMJs exhibited complete (39.4 ± 6.3%) or partial (31.3 ± 1.4%) denervation in APP+/+;SOD1G93A mice, while only 29.3 ± 4.4% remained fully innervated. However, APP ablation resulted in a significant >2-fold increase in the proportion of fully innervated NMJs (65.3 ± 2.1%, P = 0.0022) and an equally dramatic decrease in the proportion of denervated NMJs (8.6 ± 0.6%, P = 0.002) in APP−/−;SOD1G93A versus APP+/+;SOD1G93A EDL muscle. The proportion of NMJs exhibiting partial innervation was similar to that of APP+/+;SOD1G93A mice (26.1 ± 1.9%), while the total number of NMJs was also similar in APP+/+;SOD1G93A versus APP−/−;SOD1G93A EDL muscle (774 ± 48.0 compared with 800.8 ± 35.6, respectively). These results indicate that endogenous APP is detrimental either to the maintenance of innervation or to compensatory reinnervation of NMJs in SOD1G93A mice (see Discussion).

APP ablation ameliorates motor function and body mass decline in SOD1G93A mice

To determine whether the improved muscle innervation observed in APP−/−;SOD1G93A mice (Fig. 3A) was reflected in improved functional parameters, grip-strength and body mass measurements were obtained longitudinally from mice of each genotype between 57 and 120 days of age. This age range was selected to encompass pre-symptom onset through to late-stage disease in SOD1G93A mice.

As expected, APP+/+ mice displayed the strongest grip-strength values (Fig. 3B), which gradually increased as the mice aged, from 155.7 g (± 6.0) to 202.5 g (± 5.6), whereas APP ablation resulted in a modest 12.6% (± 3.0) average decrease in grip-strength compared with APP+/+ mice, consistent with previous reports (25). APP heterozygosity did not significantly affect grip-strength compared with APP+/+ mice. However, even from the earliest time point, SOD1G93A expression resulted in significantly reduced grip-strength in all three APP genotypes compared with their respective SOD1G93A−negative controls (Fig. 3B). In APP+/+;SOD1G93A+/− and APP−/−;SOD1G93A mice, grip strength began to decline significantly from 96 days and 92 days, respectively (denoted by colour-coded arrows along x-axis).

However, in APP+/−;SOD1G93A mice, grip strength remained stable until 117 days before decreasing significantly, which represents a 21–25-day delay in disease progression. Additionally, direct comparison between SOD1G93A−positive groups revealed that APP−/−;SOD1G93A mice were significantly stronger than APP+/+;SOD1G93A+/− (indicated by green asterisks in Fig. 3B) and APP−/−;SOD1G93A (indicated by yellow asterisks in Fig. 3B) groups from 113 days and 110 days, respectively.

Longitudinal analysis of body mass (Fig. 3C) revealed that APP ablation also causes a modest (10.0 ± 1.1%) reduction in body mass compared with APP+/+ mice, as previously reported (25). SOD1G93A expression also resulted in a significant decrease in body mass, which affected each APP genotype to a similar extent (10.2 ± 3.8% for APP+/+;SOD1G93A; 16.5 ± 5.0% for APP−/−;SOD1G93A; and 11.7 ± 2.9% for APP−/−;SOD1G93A). Importantly, the average maximum body mass of APP+/+;SOD1G93A and APP−/−;SOD1G93A mice peaked at 96 days and 99 days (20.15 ± 0.47 g and 20.02 ± 0.49 g, respectively). In contrast, the body mass of APP−/−;SOD1G93A mice continued to increase until 117 days, albeit to a lower maximal value (17.47 ± 0.34 g). The prolonged increase in body mass of APP−/−;SOD1G93A mice provides further evidence of delayed disease progression, or possibly delayed onset, and closely mirrors the 21-day delay observed for grip strength.

Since the decreased grip strength caused by APP ablation was accompanied by a similar reduction in body mass values, it appeared likely that this effect is directly accounted for by decreased body size. Therefore, to correct for this effect, the grip strength to body mass ratio (grip strength/body mass) was calculated for each genotype (Fig. 3D). Interestingly, although this normalization abolished any difference between APP−/− and APP+/+ mice, it revealed that the average grip strength:body mass ratio is reduced in APP+/+ mice (12.3 ± 4.5%), suggesting that their larger body mass is due to obesity, rather than a proportionate increase in skeletal muscle mass.

SOD1G93A expression caused a progressive decline of the grip-strength:body mass ratio in all three APP genotypes, in contrast to the relatively consistent values of their respective SOD1G93A−negative controls (Fig. 3D). In APP+/+;SOD1G93A+/− and APP−/−;SOD1G93A mice, the grip strength:body mass ratio declined significantly from initial values at 78 days and 71 days, respectively (denoted by coloured arrows along x-axis). Importantly, however, the grip strength:body mass ratio of APP−/−;SOD1G93A mice remained stable until 103 days, before exhibiting a significant decrease, which represents a 25–32-day delay in disease onset or progression. Additionally, direct comparison of grip strength:body mass ratios between APP−/−;SOD1G93A mice versus APP+/−;SOD1G93A+/− (green asterisks) or APP−/−;SOD1G93A (yellow asterisks) mice revealed that APP ablation confers significantly greater values from 92 days onwards (Fig. 3D). Thus, these results demonstrate that APP ablation does indeed confer functional improvement in SOD1G93A mice and significantly delays the point at which critical milestones of disease progression are reached, which is in keeping with improved muscle innervation (Fig. 3A).

APP ablation improves muscle contractile characteristics in late-stage SOD1G93A mice

To determine whether APP ablation ameliorates SOD1G93A−mediated decline in tetanic force of fast-twitch muscles, in vivo isometric muscle tension physiology was performed on tibialis anterior (TA) and EDL muscles from 120-day mice. Both of these muscles exhibit significant denervation, reduced force production, atrophy and switch to a slower (more oxidative) phenotype by late-stage disease in SOD1G93A mice (26,27). Although APP ablation did improve grip strength (reported earlier) in SOD1G93A mice, tetanic force of TA or EDL muscles was not improved (Supplementary Material, Fig. S5A and B). However, these results demonstrate that the EDL muscle is less severely
affected by SOD1<sup>G93A</sup> expression than TA muscle in all three APP genotypes at 120 days. Thus, muscles that have a similar phenotype and are innervated by similar motor neuron populations can be affected at different stages of disease progression. This may partially explain the apparent discrepancy between the improved grip strength and the lack of improved tetanic force of these two muscles (see Discussion).

Nonetheless, assessment of the contractile characteristics of TA and EDL muscles revealed a dramatic improvement in features that are known to change significantly in SOD1<sup>G93A</sup> mice (26,27). The time taken for muscles to reach maximum force (time-to-peak, TTP), and to relax to half the maximum force (1/2 relaxation time, 1/2RT) following a twitch-stimulus is prolonged in SOD1<sup>G93A</sup>-mediated disease. The TTP and 1/2RT were unaffected by APP genotype in SOD1<sup>G93A</sup>-negative TA muscle (Supplementary Material, Fig. S5C and D) and EDL (Fig. 4A and B) muscles. However, SOD1<sup>G93A</sup> expression significantly delayed TTP in TA and EDL muscles of both APP<sup>1/2</sup>:SOD1<sup>G93A</sup> and APP<sup>−/−</sup>:SOD1<sup>G93A</sup> mice. Similarly, the 1/2RT was also significantly delayed in TA and EDL muscles of both APP<sup>1/2</sup>:SOD1<sup>G93A</sup> and APP<sup>−/−</sup>:SOD1<sup>G93A</sup> mice.

In APP<sup>1/2</sup>:SOD1<sup>G93A</sup> mice, however, TTP and 1/2RT values of TA (Supplementary Material, Fig. SSC and D) and EDL (Fig. 4A and B) muscles were almost completely rescued. This demonstrates that the absence of APP prevents SOD1<sup>G93A</sup>-induced changes in contractile characteristics, possibly by preservation of fast-twitch muscle fibres, which is in keeping with the increased innervation observed in APP<sup>1/2</sup>:SOD1<sup>G93A</sup> mice (Fig. 3A). Therefore, post hoc histochemical analysis was carried out on EDL muscle sections to determine the composition of muscle fibre types.

As the EDL muscle is a predominantly fast-twitch muscle in mice, it normally contains few type-I fibres (dark ATPase staining in Fig. 4C). By late-stage disease in APP<sup>1/2</sup>:SOD1<sup>G93A</sup> muscle, clusters of type-I muscle fibres become...
readily apparent, due to reinnervation of fibres by disease-resistant slow motor neurons. However, the increased abundance of type-I fibres is dramatically abrogated in age-matched APP−/−:SOD1G93A EDL muscle and less clustering is observed. Coupled with the maintenance of contractile characteristics in APP−/−:SOD1G93A mice (Fig. 4A and B), this evidence indicates that innervation by motor neurons with faster firing properties is maintained, since muscle fibre type is highly influenced by the firing pattern of the innervating motor neuron (28,29). Therefore, APP ablation appears to delay the death of vulnerable fast-firing motor neurons, up until late-stage disease, thus the effect of APP ablation on motor neuron survival in SOD1G93A mice was investigated.

APP knockout promotes functional motor neuron survival in 120-day SOD1G93A mice

In vivo MU number estimates were obtained for EDL muscles from 120-day mice of each genotype (representative traces are shown in Fig. 5A). The number of EDL MUs (Fig. 5B) was not altered as a result of APP ablation alone (APP+/+ = 32 ± 0.5, APP−/+ = 30.9 ± 0.9 and APP−/− = 30.3 ± 0.8).

However, by late-stage disease, there was a highly significant loss of EDL MUs in SOD1G93A-positive mice (Fig. 5B) as previously shown (26,27). Importantly, in SOD1G93A mice in which APP was ablated, EDL MU survival was dramatically increased by 62.42 ± 5.69% (P = 0.0088), compared with APP+/+:SOD1G93A mice.

In order to corroborate the physiological findings described above, post hoc histological counts of motor neurons in the sciatic pool of the lumbar spinal cord (area delineated in images, Fig. 5A) was undertaken. As expected, in SOD1G93A-negative controls, the APP genotype did not significantly affect motor neuron numbers (APP+/+ = 432.69 ± 27.52, APP−/+ = 381.51 ± 15.34 and APP−/− = 373.29 ± 11.02), whereas SOD1G93A expression resulted in a highly significant loss of motor neurons in all APP genotypes (Fig. 5C). However, in agreement with the MU assessment, APP ablation significantly improved motor neuron survival by 64.86 ± 3.97% (P = 0.0021) compared with APP+/+:SOD1G93A mice. These two different techniques demonstrate that the presence of endogenous APP is detrimental to motor neuron survival in SOD1G93A-mediated disease. It is therefore possible that elevated levels of Aβ peptides and their

Figure 4. APP ablation rescues muscle contractile characteristics late-stage SOD1G93A mice. Twitch stimuli were delivered to elicit TTP contraction (A) and half-relaxation time (RT) (B) for EDL muscles from mice of each genotype (see key below bar charts for colour coding and n-values). Data are shown as mean values ± SEM. *P ≤ 0.05, **P ≤ 0.005, ***P ≤ 0.0005 (Student’s t-tests with post-hoc Holm–Bonferroni multiple-test correction). Post hoc histochemical staining was performed on a subset of EDL muscles following physiological recordings. Representative images of ATPase staining (pH 4.2) in EDL sections from four genotypes of interest (C), in which type-I muscle fibres appear dark (note: nerve fibres and capillaries also appear dark). Insets at bottom left of images show enlarged regions of interest (scale bars = 200 μm).

Figure 5. APP ablation reduces SOD1G93A-mediated loss of MUs and motor neurons at late-stage disease. (A) Upper panel shows representative physiological MU traces obtained from EDL muscles at 120 days for three genotypes of interest. Twitch amplitude is adjusted to show maximum separation between MUs and is therefore not to scale. (A) Lower panel shows representative images of post hoc Nissl staining of lumbar spinal cord sections following MUNE recordings; the dashed red line delineates the approximate boundary of the sciatic motor pool in which motor neurons were counted and insets at bottom left of each image show higher magnification within this region (scale bars = 200 μm). Bar charts show mean MUNE values (B) and histological motor neuron counts per sciatic pool (C) for each genotype investigated in this study. Key below bar charts shows colour coding for each genotype and n-values for MUNE data; n = 4 for histological counts. Data represents mean values ± SEM. *P ≤ 0.05, **P ≤ 0.005 (one-way ANOVA with post-hoc Holm–Bonferroni multiple-test correction).
accumulation within motor neurons and/or astrocytes may underlie the toxic contribution of APP to motor neuron degeneration in SOD1<sup>G93A</sup> mice. Such a possibility is supported by the recent observation of motor neuron loss in mouse models of AD (22).

**APP knockout does not extend lifespan of SOD1<sup>G93A</sup> mice**

Surprisingly, despite the significant delay in disease onset or progression and reduction in motor neuron degeneration induced by APP ablation in SOD1<sup>G93A</sup> mice, analysis of lifespan of APP<sup>+/+</sup>:SOD1<sup>G93A</sup> and APP<sup>−/−</sup>:SOD1<sup>G93A</sup> mice did not reveal any significant extension in survival (Supplementary Material, Fig. S6). This finding may suggest that the detrimental effects of APP observed in SOD1<sup>G93A</sup> mice contribute to the onset and early phases of disease, rather than later stages when disease progression is very dramatic.

**DISCUSSION**

The results of this study demonstrate that APP exerts a detrimental role in disease progression in the SOD1<sup>G93A</sup> mouse model of ALS. Together with previous reports of APP upregulation in muscle (18) and the detection of intracellular Aβ in motor neurons (30) of both sporadic and familial ALS cases, these findings strongly suggest that APP may contribute to pathology in humans, irrespective of the initial trigger. These results also indicate that there may be two separate components to the pathological contribution of APP. First, the upregulation of APP in type-IIb muscle fibres, which begins concomitantly with NMJ dysfunction and correlates with muscle fibre atrophy and denervation. Secondly, the increased amyloidogenic processing of APP in the CNS may also contribute directly to MN degeneration. However, from the present study, it is difficult to separate these two possible components, which may indeed be inter-dependent.

Although APP (or its homologue, APLP2) is required for NMJ formation during development (31), overexpression of APP in cultured human myocytes inhibits NMJ formation (19). Furthermore, during NMJ development, APP mediates localized activation of caspase-6 and destruction of exuberant motor axon terminals, via the DR-6 receptor (32). Therefore, it is possible that increased expression of APP in selective muscle fibres of SOD1<sup>G93A</sup> mice could have a negative impact on the compensatory nerve terminal sprouting and NMJ formation that occurs in response to the initial denervation of muscle fibres. This may explain the atrophy and denervation of APP-positive muscle fibres observed in this study. Indeed, SOD1<sup>−/−</sup>-mediated loss of NMJ innervation and transformation of muscles to a slower phenotype is dramatically abrogated in the absence of APP. Interestingly, the increased MU number estimates for the EDL muscle and increased sciatic pool motor neuron counts, along with the reduced number of type-I muscle fibres and diminished fibre-type clustering at 120 days, strongly suggest that rather than enhancing sprouting and reinnervation by the remaining motor neuron axons, APP ablation in SOD1<sup>G93A</sup> mice appears to maintain neuromuscular contacts. However, in order to conclusively demonstrate whether the improved innervation observed in APP<sup>−/−</sup>:SOD1<sup>G93A</sup> mice is due to increased maintenance or increased compensatory sprouting of motor axon terminals will require future studies.

Surprisingly, despite improved innervation and overall motor function, muscle force of the two specific hind-limb muscles examined was not improved by APP ablation in SOD1<sup>G93A</sup>-positive mice. However, this may be accounted for by evidence that muscle fibres are themselves a target of SOD1<sup>G93A</sup>-mediated pathology (8,9). Thus, muscle fibre damage could prevent improved contractile force, despite improved innervation. Nonetheless, longitudinal analysis of grip strength did reveal a significant improvement in overall motor function at the whole-animal level.

The upregulation of APP following denervation in WT muscle suggests that this response may constitute a physiological program to prioritize re-innervation of type-I and type-IIa muscle fibres over the stronger, but more readily compromised type-IIb fibres. Fast-firing motor neurons (13,14) and their target type-IIb muscle fibres (15) are preferentially affected in ALS. However, the susceptibility of these motor neurons may not entirely account for this effect. Rather, this study suggests that the preferential denervation and loss of these muscle fibres may be a bi-directional process. During disease progression, inhibition of compensatory NMJ formation by APP could reduce the supply of target-derived neurotrophic factors, thereby depriving stressed SOD1<sup>G93A</sup> motor neurons of pro-survival cues and potentially accelerating their death. Furthermore, the upregulation of Nogo A/B in muscle fibres, which is well characterized as a myelin-associated inhibitor of axonal regeneration (33), has been shown to be induced by APP overexpression in cultured myocytes (34) and therefore could inhibit NMJ reinnervation. It should be noted, however, that NogoA/B (also known as reticulon-4/RTN-4) may promote survival of motor neurons in mutant SOD1 mice via regulation of endoplasmic reticulum function (35), thus its effects in ALS are not clear and likely depend on expression levels of different isoforms and their subcellular localization. Nonetheless, APP may contribute to several pathways that restrict reinnervation of muscle fibres following NMJ dysfunction.

Another mechanism by which APP may contribute to muscle pathology in SOD1<sup>G93A</sup> mice is via its amyloidogenic processing. A generalized release of Aβ<sub>42</sub> from muscle fibres, although capable of inducing synaptotoxicity, would be unlikely to replicate the specific pattern of denervation observed in SOD1<sup>G93A</sup> mice. Indeed, Aβ<sub>42</sub>-X<sub>42</sub> levels in muscle were found to be below the detection threshold of the highly sensitive ELISA assay employed in this study, suggesting that this is not the case. However, it is possible that Aβ<sub>42</sub> has a more localized effect at the NMJ. The N-terminus cleavage products of APP, including Aβ<sub>42</sub>, have been shown to be anterogradely transported along axons (36), potentially leading to presynaptic accumulation of Aβ<sub>42</sub>, thereby directly contributing to NMJ pathology. The extremely small volume of nerve terminals, relative to that of the entire muscle, would almost certainly have masked any such effect by the ELISA analysis; future electron microscopy studies may be able to resolve this issue.

The increased concentration of Aβ<sub>42</sub> peptides, together with the presence of Aβ<sub>42</sub> peptides in both motor neurons and
astrocytes, in late-stage SOD1\textsubscript{G93A} spinal cord suggests that amyloidogenic processing of APP may directly contribute to motor neuron death. The elevation of A\textsubscript{β} peptide levels in SOD1\textsubscript{G93A} mice reported in this study may reflect the conditions of oxidative stress that abound in the CNS at this stage; however, the time-course and mechanisms underlying this potentially pathologic process remain to be investigated. Interestingly, the susceptibility of cortical cholinergic neurons to A\textsubscript{β}\textsubscript{42} is well established in AD and several recent studies in different AD mouse models have now shown that elevated A\textsubscript{β}\textsubscript{42} levels are directly toxic to spinal motor neurons (22). Although these AD mouse models accurately mimic the formation of A\textsubscript{β} plaques, as occurs in human AD brains, death of cortical neurons is scarce by comparison. Therefore, the finding of widespread spinal motor neuron death in these AD models demonstrates the susceptibility of motor neurons to A\textsubscript{β}\textsubscript{42}.

Although evidence exists that supports a possible link between APP (or its cleavage products) and motor neuron degeneration, this study demonstrates that genetic ablation of endogenous APP ameliorated multiple aspects of disease progression in SOD1\textsubscript{G93A} mice, including functional, physiological and histological parameters. While ablation of APP did not result in a significant extension of survival in the SOD1\textsubscript{G93A} mouse model of ALS, the translation of these disease modifying effects to a clinical setting could potentially confer a dramatic improvement to the quality of life of patients with ALS. Together with previous findings, therefore, our results suggest that targeting APP may be helpful in the design of a disease-modifying approach for ALS.

**MATERIALS AND METHODS**

**Antibodies**

The following primary antibodies were used during this study: monoclonal rabbit antibody, raised against human amyloid precursor protein (clone Y188, Epitomics); mouse monoclonal antibodies, raised against human superoxide dismutase-1 (SOD1; clone SD-G6, Sigma); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) conjugated to horseradish peroxidase (HRP) (Abcam, ab9482), neurofilament heavy chain (NFH), synaptic vesicle antigen-2 (SV2), β-III tubulin (TUJ1, Covance) and isoform-specific myosin markers (clones 2H3, SV2 and BF-F3, BF-35, SC-71 and BA-D5) were all obtained as supernatants from the Developmental Studies Hybridoma Bank (DSHB), developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA); polyclonal rabbit antibody raised against rodent β-amyloid\textsubscript{3–16} (Abcam, ab14220) and the β-amyloid\textsubscript{42} neo-epitope (Ab\textsubscript{42}; Invitrogen 44-344). Secondary antibodies employed in this study included: HRP-conjugated goat anti-rabbit and goat anti-mouse (both from Thermo Scientific), biotinylated goat anti-rabbit (Jackson ImmunoResearch Laboratories) and AlexaFluor-488-conjugated donkey anti-rabbit and AlexaFluor-568-conjugated goat anti-mouse (both from Invitrogen). Tyramide signal amplification was employed for immunofluorescent detection of A\textsubscript{β}\textsubscript{42} according to the manufacturer’s instructions (Perkin Elmer, NEL741).

**Animals**

Heterozygous high-copy SOD1\textsubscript{G93A–1Gur} transgenic mice (2) and WT littermates, originally obtained from Jackson Laboratories and maintained on a C57Bl6/SJL background, were used for histological and biochemical characterization of APP expression in muscle and spinal cord tissue. Additionally, WT mice were used to histologically characterize the APP response in muscle following sciatic nerve ligation, which was performed under deep anaesthesia (isoflurane), using sterile surgical techniques.

For knockout studies, male SOD1\textsubscript{G93A–/–} mice (described above) were crossed with APP\textsubscript{+/–} mice (25), on a C57Bl6/BALB/c background, kindly provided by S. Soriano. This cross-yielded APP\textsubscript{−/−} and APP\textsubscript{+/−}:SOD1\textsubscript{G93A−/−} genotypes which were then back-crossed to produce APP\textsubscript{+/+}, APP\textsubscript{−/−}, APP\textsubscript{+/−}:SOD1\textsubscript{G93A−/−}, APP\textsubscript{−/−}:SOD1\textsubscript{G93A−/−} genotypes. Genotype of progeny was confirmed by PCR using primers for endogenous APP (Forward\textsuperscript{Present}, 5′-CCA TTG CTC AGC GGT GCT G-3′; Forward\textsuperscript{Excised}, 5′-CAG CTC TAT ACA AGC AAA CAA G-3′; and Reverse: 5′-CTG CTC CAG GTG CTC G-3′) as well as for endogenous SOD1 (Forward 5′-CTA GGC CAC AGA ATT GAA AGA TCT-3′ and Reverse 5′-GTA GGT GGA AAT TCT AGC ATC-3′) and transgenic human SOD1 (Forward 5′-CAT CAG CCC TAA TCC ATC TGA-3′ and Reverse 5′-CGG GAC TAA CCA TAA CAA TCA AAC TGA-3′). To eliminate variation caused by sex differences and reduce the number of mice required for this study, only age-matched female mice (from the same litter where possible) were used for longitudinal behavioural analysis (n ≥ 19 per genotype), muscle physiology (n ≥ 5 per genotype), post hoc histology (n ≥ 4 per genotype) and survival (n = 13 per genotype) studies. Post hoc correlation analysis of longitudinal behavioural data was performed to ensure that the predicted percentage of BALB/c in the genetic background of each animal did not influence the experimental outcome, which was found not to be the case (Max coefficient of correlation < 0.05). The similarity between survival of APP\textsubscript{+/+}:SOD1\textsubscript{G93A−/−} and APP\textsubscript{−/−}:SOD1\textsubscript{G93A−/−} genotypes also indicates that genetic background did not affect the results of this study. Male mice were used for breeding and for biochemical (ELISA and western blot) analysis. Animals were maintained in temperature and humidity controlled environment with a 12 h light–dark cycle, and ad libitum access to food and water. All procedures and experiments involving animals were carried out under License from the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and following ethical approval from UCL Institute of Neurology.

**Longitudinal motor function and body mass analysis**

Grip strength measurements were recorded every 3–4 days using a Bioseb\textsuperscript{TM} force gauge according to the manufacturer’s instructions. Briefly, mice were placed on a horizontal grid, allowing all four limbs to engage and then pulled by the base of the tail against the direction of the force gauge until the animal released the grid. An average of four maximum readings was obtained every 3–4 days for each animal.
between the age-range of 57–120 days, values obtained were pooled for each of the six genotypes. Body mass was recorded at the same time as grip-strength measurement (every 3–4 days) over the same age range (57–120 days). Again, average values for each of the six genotypes were pooled. The point at which maximum body mass was reached was used as a milestone for disease progression. Grip strength and body mass values were used to calculate the grip strength:body mass ratio for individual animals which were pooled for each genotype.

In vivo isometric muscle tension physiology
At a designated time point of 120 days (selected to be representative of late-stage disease, while avoiding complete paralysis and difficulties anesthetizing animals at actual disease end-stage), isometric muscle tension physiology was performed on female mice of each of the six genotypes investigated in this study, as previously described (26,27). Briefly, under deep anaesthesia (4.5% chloral hydrate, i.p.), hindlimbs were immobilized and the distal tendons of the TA and EDL muscles of both hindlimbs were exposed and consecutively attached to force transducers in parallel. Sciatic nerves were exposed bilaterally, at mid-thigh level, severed and the distal stumps placed in contact with stimulating electrodes. TTP and half-relaxation times (1/2 RT) were recorded following square wave stimuli of 0.02 ms duration. The time taken to reach maximal contraction (TTP) and to relax again to half-maximal values (1/2 RT) was determined for individual muscles using Picoscope™ software and averaged for each genotype. Tetanic contraction was induced by delivering trains of stimuli at 40, 80 and 100 Hz for 2.5 s duration, to induce the maximal contractile force. Again, values for individual muscle were determined using Picoscope software, averaged for each genotype. EDL MU number estimates (MUNE) were determined by gradually increasing the amplitude range of amplitudes was counted for individual muscles and averaged for each genotype.

Tissue processing and histology
Immediately following muscle physiology recordings, while animals remained under terminal anaesthesia, unilateral TA and EDL muscles were removed, weighed, embedded in Tissue-Tek O.C.T. compound and flash-frozen in isopentane super-cooled in N2(l). In animals that had not undergone physiological recording, terminal anaesthesia was induced by sodium pentobarbital, mice were then trans-cardially perfused with 10 ml saline followed by 20 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer and 0.9% NaCl. The remaining TA and EDL muscles, along with gastrocnemius muscle and lumbar spinal cord, were then removed, post-fixed in 4% PFA in tris-buffered saline (TBS, pH 7.6) and cryoprotected in 10% then 20% sucrose in TBS at 4°C o/n. Tissue was then embedded in Tissue-Tek O.C.T. compound and frozen on dry-ice. Muscles from male SOD1G93A and WT mice were processed in the same manner, as were muscles from 14-day post-sciatic nerve axotomy mice. Muscle and lumbar spinal cord tissue for western blot and ELISA experiments were rapidly removed from age-matched male mice of each genotype, immediately following cervical dislocation, briefly washed in ice-cold PBS, weighed and then flash-frozen in a 1.8 ml cryotube (Nunc) in N2(l). Samples were stored at −80°C until use.

Muscle histology
Transverse flash-frozen muscle sections were cut on a cryostat (12 μm thickness) and histochemical ATPase staining was performed at pH 4.2 (n = 4 per genotype). Transverse frozen sections (12 μm) were also cut from perfused TA and gastrocnemius muscles which were immunolabeled for APP (diluted 1:1000 in TBS + 2% normal goat serum, hereafter called TBS+), followed by biotinylated goat anti-rabbit—the signal was then amplified using Vectastain ABC Elite kit (Vector Labs) and developed using diaminobenzidine (ImmPACT DAB; Vector Labs) as a chromogen. Type-Ib-specific myosin (clone BF-F3, diluted 1:10 in TBS+) was immunofluorescently labelled in unfixed sections, followed by goat anti-mouse-AlexaFluor488 (diluted 1:500 in TBS+). Muscle fibre CSA was measured in 84-day SOD1G93A and WT gastrocnemius muscle, following APP immunohistochemical labelling. Values shown in Figure 1E represent average CSA sampled from 3529 SOD1G93A MGC fibres (n = 3) and 2281 WT MGC fibres (n = 3). Fibre-type-specific myosin isoforms were immunolabelled in unfixed sections with combinations of the following monoclonal antibodies: clone BF-F3 (type-Ib), clone BF-35 (all fibres except type-Ih), clone SC71 (type-Ih) and clone BA-D5 (type-I). Primary antibodies were diluted 1:10 in TBS+ and immunofluorescently labelled using goat anti-mouse IgG-AlexaFluor488 and/or goat anti-mouse IgG-AlexaFluor568 (both from Invitrogen), diluted 1:500 in TBS+.

NMJ innervation counts
Frozen longitudinal EDL muscle sections were cut on a cryostat (20 μm thickness). Alternate sections from entire muscles were first blocked using the M.O.M. kit (Vector Labs), to prevent non-specific secondary antibody binding to mouse tissue. Sections then underwent double immunofluorescent labelling for NF (diluted 1:10 in TBS+; clone 2H3) and SV-2 (diluted 1:10 in TBS+), which were simultaneously detected using goat anti-mouse-AlexaFluor488; post-synaptic acetylcholine receptors were labelled with α-bungarotoxin conjugated to rhodamine. Only alternate sections were analysed to preclude counting the same NMJ more than once. NMJs were scored according to whether there was complete co-localization of pre-and post-synaptic markers (fully innervated), partial co-localization (intermediate innervation) or only post-synaptic labelling (fully denervated). Since the objective was to determine the effect of APP deficiency on muscle fibre innervation in SOD1G93A-mediated disease, only SOD1G93A-positive genotypes were included in this analysis (n = 4 per genotype).
**Spinal cord histology**

Frozen transverse sections from lumbar spinal cords, encompassing spinal levels T11-L6, were cut on a cryostat (20 μm thickness). For histological motor neuron counts, Nissl staining was carried out on sections using galloycyanine. Large (>15 μm diameter) alpha motor neurons that had a visible nucleolus and were localized to the sciatic motor pool between L3 and L5 were counted bilaterally from every third section, to preclude counting the same motor neuron more than once. A minimum of 40 sections from each spinal cord were counted (n = 4 per genotype). All counts were performed by a blinded observer. Additionally, lumbar spinal cord sections from APP\(^{+/+}\) (WT) and SOD1\(^{G93A}\) mice were immunofluorescently labelled to detect endogenous (murine) β-amyloid (rabbit polyclonal anti-\(\beta\)-\(\beta\)3–16) and the \(\beta\)42 cleavage-site specific neo-epitope (rabbit polyclonal anti-\(\beta\)42), which was then labelled using goat anti-rabbit-biotin (Vector Labs), followed by HRP conjugation (Vectastain ABC kit, Vector Labs) and tyramide signal amplification. Sections were also labelled with polyclonal goat anti-choline acetyltransferase or monoclonal mouse anti-β-III tubulin or mouse anti-GFAP (Cy-3 conjugate, Sigma); secondary labelling, using donkey-anti-goat-Alexafluor568 (applied prior to tyramide labelling followed by a second blocking step with 5% normal goat serum in TBS+) or goat anti-mouse-AlexaFluor568 (applied after completion of tyramide labelling), was performed sequentially to avoid non-specific binding. Results were imaged using a Leica DMR epifluorescence microscope.

**ELISA quantification of soluble A\(\beta\)\(_{40}\) and A\(\beta\)\(_{42}\) levels**

Commercial kits for the detection of human/rodent A\(\beta\)\(_{40}\) (Wako, #294-62501) and high-sensitivity human/rodent A\(\beta\)\(_{42}\) (Wako, #292-64501) were used according to the manufacturer’s instructions. *Gastrocnemius* muscle and lumbar spinal cord tissue from 120-day male APP\(^{+/+}\), APP\(^{+/−}\); SOD1\(^{G93A}\) and APP\(^{−/−}\);SOD1\(^{G93A}\) (negative control) mice was thawed on ice and homogenized in 40-vols of 0.2% diethylamine (DEA) in 50 mM NaCl solution (pH10), using a Polytron PT 1200 ultrasonic homogenizer for 2 min on ice. After 1 h at 4°C, samples were transferred to polallomer tubes (Beckman #362333) and centrifuged at 133000g for 45 min at 4°C. The supernatant was then collected, neutralized with 1/10 volume of 0.5 M Tris/HCl (pH6.8), frozen on dry ice and stored at −80°C until analysed. Upon thawing on ice for analysis, protein concentration of each sample was determined using the DC protein assay (BioRad) so that ELISA results could be normalized to total protein content. Samples (n = 3 per genotype) were run in duplicate and results are displayed as mean ± SD.

**Western blots**

Frozen spinal cord and muscle tissue samples were homogenized in 5 volumes of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% Triton X-100, containing protease inhibitor cocktail, Sigma), on ice, using a Polytron PT 1200 ultrasonic homogenizer. After 1 h at 4°C, samples were centrifuged to remove cell debris and supernatants were collected. Protein concentration was determined using DC protein assay (Biorad) and samples were then aliquoted and stored at −80°C until use. Samples were separated under reducing and denaturing conditions on 4–20% Tris-glycine gels (Biorad), then transferred to nitrocellulose membranes, which were immunoblotted with the following antibodies (diluted in TBS + 0.1% Tween-20 + 2% milk): anti-APP (1:1000, clone Y-188), anti-SOD1 (1:500, clone SD-G6) and anti-GAPDH-HRP conjugated (1:2000) as a loading control. Protein bands were labelled with HRP-conjugated Goat anti-Rabbit (1:2000) or Goat antimouse (1:1000) and visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), captured using X-ray film (Fuji), which were then scanned. Antigenic band densitometry was determined using Alpha Innotech software and normalized to loading controls. All immunoblots were run at least twice (n = 2 samples per age/genotype).

**Statistical analysis**

For longitudinal data, one-way analysis of variance (ANOVA) was conducted for each time point to determine the differences between genotype and Bonferroni’s *post hoc* analysis was employed for multiple-test correction; statistically significant decline from initial values within each group was determined using a series of Student’s *t*-tests, with Holm–Bonferroni multiple-test correction. Differences in innervation were determined by one-way ANOVA. Motor neuron counts and MU number estimates were analysed by one-way ANOVA, followed by Holm–Bonferroni multiple-test correction. Muscle physiology data were compared by Student’s *t*-test with Bonferroni multiple-test correction. ELISA and western blot quantification are shown as mean ± SD and statistical differences between groups were determined by Student’s *t*-test.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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*Conflict of Interest statement.* None declared.

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