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Unaffected motor endplate occupancy in eye muscles of ALS G93A mouse model

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1. ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal neurodegenerative disorder characterised by selective loss of motor neurons with accompanying muscle paralysis and respiratory failure. Despite progressive paralysis in trunk and extremity muscles, disturbed eye motility is not a hallmark of ALS. Extraocular muscles (EOMs) of terminal ALS patients show far less morphological signs of disease than their limb muscles. One of the earliest signs of the disease in the transgenic G93A SOD1 mouse model of ALS is loss of motor neuron contact at the neuromuscular junctions (NMJ) in limb muscles. We used immunohistochemistry to identify NMJs and evaluate innervation in EOMs and limb muscles of G93A mice. In G93A limb muscles, loss of axonal contact was seen in 6-82 % of the NMJs. On the contrary, the degree of endplate occupancy in the EOMs did not differ between transgenic mice and wild-type controls. We propose that EOM-specific properties make these muscles more resistant to the underlying pathophysiological process of ALS and that the EOMs are a useful model to advance our understanding of ALS.

2. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a presently incurable neurodegenerative disease whose detailed pathophysiology is poorly understood. ALS is characterised by progressive muscle paralysis that eventually leads to death from respiratory failure. The vast majority of ALS cases are 'sporadic' (SALS) and usually of unknown cause (1-3). In contrast, approximately 10% of the ALS cases are hereditary, familial amyotrophic lateral sclerosis FALS(4) and in 12 to 23% of these cases, the disease is associated with mutations in the gene that encodes the enzyme superoxide-dismutase 1 (SOD1)(1,5). SOD1 is a ubiquitous enzyme located mainly in the cytoplasm where it plays an important role in the conversion of superoxide into the less reactive hydrogen peroxide (6). Although the mechanisms behind the disease are not fully understood, it has been established that SOD1 mutations lead to a 'toxic gain-of-function' (3).

More than 166 disease-causing SOD1-mutations have been discovered to date (1), with the D90A mutation being the most common (1). Several transgenic mice

models over-expressing D90A, G127X, G93A and G85R, known human SOD1 mutations, have been generated to study ALS. Of these, the first generated G93A is the most commonly used model to study the pathophysiology of ALS (7).

The histopathological hallmark of ALS are the loss of lower motor neurons in the anterior horns of the spinal cord with accompanying sclerosis of the lateral corticospinal tracts (8). Hence, the main focus of ALS research on the motor neurons and their neighbouring cells in the central nervous system. However, a growing body of evidence indicates that peripheral processes in the muscle also play an important part in the early stages of ALS (9-13). In particular, axonal withdrawal from the neuromuscular junction (NMJ) seems to occur very early in the course of the disease before death of motor neurons can be detected in the spinal cord (14). This early withdrawal of motor neuron axons at the NMJs occurs before the animals start to show loss of motor function, and is later followed by a reduced number of axons in the ventral roots of the spinal cord. Loss of motor neurons is detected at an even later stage, suggesting that the loss of neurons is one of the last effects in a long chain of events (13). Also, in the same study, samples from an ALS-patient who died of early and unrelated reasons showed similar histological changes (13) leading the authors to use the term "dying back" to describe this process of retrograde denervation.

Gaze problems have been reported in some ALS patients, especially those whose lifespan has been extended with the aid of mechanical ventilation (15-18). However, disturbed ocular motility is not a hallmark of ALS in contrast to the involvement of other cranially innervated muscles such as those involved in speech and swallowing (19). Autopsy data of 27 ALS patients has revealed that the oculomotor nuclei are usually well preserved, only occasionally showing inclusions consistent with ALS. Pathological signs of ALS in the oculomotor nuclei were more frequently seen in patients with concomitant opthalmoplegia or ALS-related dementia. Still, actual motor neuron loss was uncommon, even in patients with opthalmoplegia (20). We have recently shown that although morphological signs of denervation and re-enervation (21), such as fibre grouping, abnormal fibre size and increased connective tissue, may be present in the EOMs of terminal ALS patients, these muscles are much less affected than their limb muscles (22).

In the present study, we have investigated whether signs of axonal withdrawal from the NMJ can be detected in the EOMs of G93A mice and be correlated to the process occurring in their limb muscles, as shown by alpha-bungarotoxin and antibodies against synaptophysin and neurofilament. Alpha-bungarotoxin is a well-known NMJ marker that selectively binds to acetylcholine receptors located in the sarcolemma of muscle fibres (23). Synaptophysin is a synaptic vesicle glycoprotein that is expressed in practically all synapses (24) and extensively used in quantitative studies of synapses (25). Neurofilament is present in neurons, and particularly abundant in the axons of neurons (26).

3. MATERIALS AND METHODS

3.1 Specimens

EOM and lower limb muscles (extensor digitorum longus, tibialis anterior, gastrocnemius and soleus) were collected from 7 transgenic mice (age 42 days – 148 days) expressing human G93A SOD1 (7) and 6 wild-type (Wt) controls (age 42 days – 116 days). The original G93A-mice were obtained from Jackson Laboratories (Bar Harbour, Maine, USA) and backcrossed with C57/BL6 BomTac for > 20 generations. The experiments and animal handling were carried out in accordance with the European Communities' Council Directive (86/609/EEC) and with consent from the Swedish central committee for animal testing (CFN).

Muscle samples were collected directly after the animals were sacrificed with an intraperitoneal injection of pentobarbital. Muscle samples were mounted on cardboard with Tissue-Tek (Sakura; Zoeterwoude, Netherlands), rapidly frozen in propane chilled with liquid nitrogen and stored at -80°C until use. Samples were either cross- or longitudinally sectioned. Serial, 8 micrometer-thick sections covering the whole length of the four recti EOMs were cut, whereas for each limb muscle, approximately 80 serial sections were processed and analysed. Sectioning was carried out at -23°C using a Reichert Jung cryostat (Leica, Nussloch, Germany).

3.2 Immunohistochemistry

In brief, the sections were left to dry in room temperature for five minutes, then placed in PBS buffer, and thereafter incubated with goat normal serum for 15 minutes. Then, the sections were incubated overnight with a buffered solution containing both primary antibodies against neurofilament protein (1:500) (subunit 160 kD Dako; Glostrup, Denmark) and against synaptophysin (1:500) (SY38 Boenhringer Mannheim Biochemica, Indianapolis, Indiana, USA) at +4°C. The following day, the samples were washed in PBS buffer and incubated with 1:20 goat normal serum at room temperature for 15 minutes. Thereafter, the samples were incubated with a buffered solution containing goat-anti-mouse (Invitrogen, USA) or donkey-anti-mouse (Jackson Immunoresearch, USA) secondary antibodies and alpha-bungarotoxin (1:600) (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at +37°C. The sections were washed in buffer and covered with Vectashield (Vector Laboratories; Burlingame, California, USA) and stored at +4°C until analysis. For negative controls, additional sections were treated as above but the primary antibody was omitted. In addition, one to two sections for each specimen were stained with haematoxylin and eosin.

3.3 Analysis

The muscle sections were viewed and photographed with a Nikon microscope (Eclipse, E800; Nikon, Tokyo, Japan) equipped with a digital camera (Spot RT color; Diagnostic instruments, Sterling Heights, MI, USA). Care was taken to correctly identify the true EOMs, i.e. the m. retractor bulbi was excluded, as this muscle does not share the EOM allotype (27). Negative controls showed

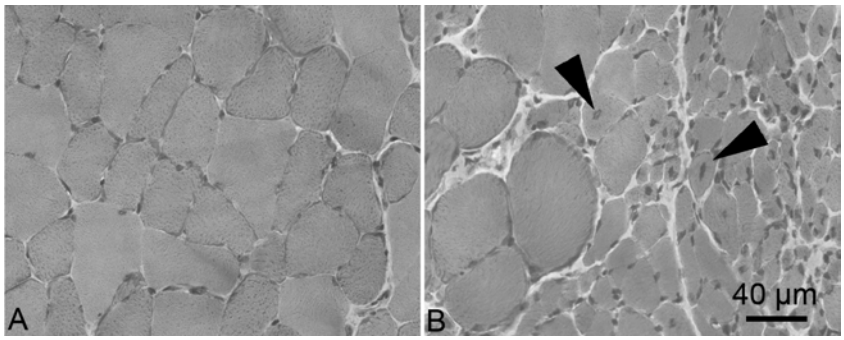

Figure 1. HE staining of limb muscles in Wt (A 116 days old) and G93A mice (B 133 days old). Note the wide variation in the size of the muscle fibres and the rounded shape of their cross-section. Several fibres with central nuclei (white arrows) can be seen, a sign of recent or on-going regeneration.

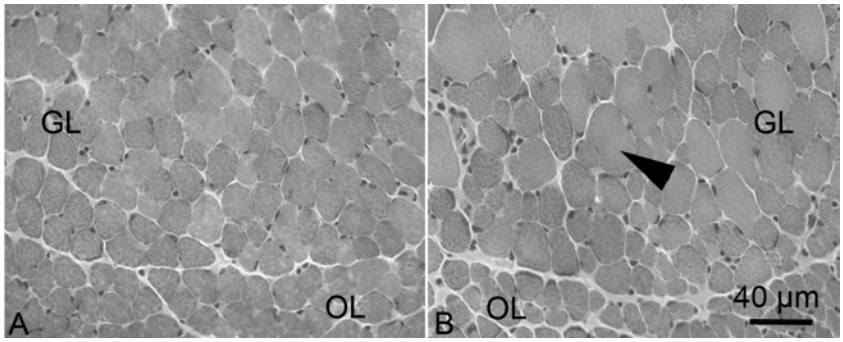

Figure 2. HE staining of EOM in Wt (A 128 days old) and G93A (B 133 days old) mice. The normal morphology of the EOMs is quite different from that of limb muscles and is characterised by small, rounded fibres with significantly more capillaries and peripheral nerves. Also, EOM have two distinct layers of muscle fibres, the global layer (GL), facing the eye, and the orbital layer (OL) with smaller fibres, facing the bony orbit. In general, the transgenic and control EOMs had a similar morphology with peripheral nuclei and modest connective tissue. However, in the G93A sample there was wider range of fibre sizes. The arrowhead marks a muscle fibre of slightly larger-than-normal diameter.

only faint, unspecific immunofluorescence, whereas strong signalling was present in the other sections when the appropriate wavelength was applied. On each muscle section, the total number of NMJs present was counted and each NMJ classified as either 'innervated' or 'denervated' by examining them at x20 and x40 magnification.

NMJs showing staining with both synaptophysin/neurofilament and alpha-bungarotoxin were classified as 'innervated'. NMJs were classified as 'denervated' when there was a clear area labelled with alpha-bungarotoxin but no adjacent or overlapping staining with antibodies against synaptophysin/neurofilament. The majority of the NMJs exhibited either a distinct and nearly total co-staining of alpha-bungarotoxin and synaptophysin/neurofilament or a complete lack of nearby synaptophysin/neurofilament staining - it was uncommon to see NMJs in an 'intermediate' state, showing only fragmentary staining with synaptophysin/neurofilament. Such NMJs were classified as innervated.

We used both longitudinal and cross sections. In order to accurately decide whether a cross-sectioned NMJ was innervated or not, it was necessary to follow the NMJ for at least four consecutive slides (32 micrometers), as the surface of the fibre stained with alpha-bungarotoxin could occasionally extend above and below the area stained with synaptophysin. Therefore, cross-sectioned NMJs were not included in the study material unless they could be traced and evaluated in four consecutive sections.

3.4 Statistical Analysis

P-values were calculated in Statview 4.5 (Abacus Concepts, Piscataway, New Jersey, USA), using an unpaired t-test. P-values were calculated to discern whether the difference between G93A-limbs and Wt-limbs were statistically significant, and whether G93A-EOM and Wt-EOM showed statistically significant differences in denervation.

4. RESULTS

Limb muscles, (Figure 1), from G93A mice showed morphological signs clearly consistent with ALS: hypertrophic fibres alongside atrophic fibres, increased connective tissue, fatty-tissue replacement, central nuclei and rounding of muscle fibres, whereas in the controls, no such changes could be detected. In the EOMs of transgenic mice, any morphological signs of muscle pathology mentioned above were much less prominent or absent (Figure 2). Muscle fibre size variation appeared slightly larger in EOMs of transgenic animals compared to controls

Table 1. Specimens

Age (Days)	Genotype	Muscle sample	Total No of NMJ (n)	Denervated NMJ (n)	Percentage denervated (%)
148	G93A	EOM	56	2	3,6
		Limb	133	50	37,6
148	G93A	EOM	130	1	0,8
		Limb	89	44	49,4
130	G93A	Limb	57	28	49,1
120	G93A	EOM	61	1	1,6
		Limb	55	45	81,8
119	G93A	EOM	93	0	0,0
100	G93A	EOM	64	5	7,8
		Limb	122	26	21,3
42	G93A	EOM	82	0	0,0
		Limb	148	9	6,1
116	Wt	EOM	69	0	0,0
		Limb	80	3	3,8
110	Wt	EOM	67	0	0,0
		Limb	64	1	1,6
110	Wt	EOM	117	5	4,3
		Limb	59	6	10,2
105	Wt	EOM	101	2	2,0
		Limb	103	9	8,7
81	Wt	EOM	120	3	2,5
		Limb	119	3	2,5
42	Wt	EOM	106	0	0,0
		Limb	59	0	0,0
Total	G93A	EOM	486	9	1,9[1]
		Limb	604	202	33,0[2]
	Wt	EOM	580	10	1,7[1]
		Limb	484	22	4,5[2]

Abbreviations: Wt - wild-type, EOM – extraocular muscles, NMJ – neuromuscular junction. Individual statistics for each specimen studied, describing age, genotype, muscle type, total number of neuromuscular junctions and total number of denervated neuromuscular junctions. [1]P= 0,569 for Wt-EOM vs G93A-EOM. [2]P= 0,0071 for Wt-Limb vs G93A-limb

(data not shown), similarly to that found in human samples (22) but central nuclei, fatty-tissue replacement and increased connective tissue were not present.

NMJs could easily be identified on the basis of alpha-bungarotoxin staining located on the surface of muscle fibres and were present in all specimens studied (Table 1, Figure 3 and Figure 4). In the limb muscles of transgenic G93A mice, 202 out of 604 NMJs (33%) were denervated whereas in the limbs of the controls, only 22 out of 484 NMJs (4,6%) were classified as denervated. The difference between these two groups was statistically significant (p=0.0071 for G93A-limbs vs Wt-limbs). In contrast, denervated NMJs were rarely seen in the EOMs of either transgenic or control mice. In transgenic mice, only 9 out of 486 EOM-NMJs (1,9%) were classified as denervated and in controls, 10 out of 580 NMJs (1,7%) were classified as denervated. Hence, there was no statistically significant difference between the EOM of transgenic and Wt mice with respect to denervated NMJs (p=0,569 for G93A-EOM vs Wt-EOM).

5. DISCUSSION

The present study has confirmed previous reports (13,14) of loss of contact between motor neurons and muscle fibres in the limb muscles of G93A-mice. In contrast, there was no statistically significant difference in the degree of loss of NMJ occupancy between the EOMs of transgenic or control mice in the advanced, manifest stages of the disease. Earlier studies on denervation on various SOD1 mice models included a separate category of intermediate NMJ innervation between 'denervated' and 'innervated' where 'partial overlap between end-plate and terminal' was seen (13). We opted to leave out this category and classify the NMJs as either 'innervated' or 'denervated', to minimize the impact of subjective judgement on the classification of NMJs. Even in normal, healthy NMJs, the area stained with alpha-bungarotoxin (representing the postsynaptic part of the NMJ) extends outside of the area labelled with antibodies against synaptophysin (representing the presynaptic part of the NMJ). Therefore, it may be difficult to objectively decide what extent of overlap should be assigned to the 'innervated' or the 'intermediate' category. Furthermore, we also had to take into account the morphology of the so-called 'en-grappe' motor end plates that are normally present in the multiply innervated EOMs (28). 'En-grappe' motor end plates consist of a chain of small, dot-like synapses along the fibre and they could have been misinterpreted as 'intermediate' because of their thin axons. Previous studies using G93A-mice have reported varying percentages of denervated NMJs in the limb muscles at the late stages of disease, ranging between 20-70% at approximately 120 days, with a majority being in the higher end of the range. Studies that included the intermediate category typically classified 10% as 'intermediate' in the later stages of the disease, (13, 29-32). Our results concerning the degree of denervation in limb muscles are within this range and indicate that the criteria used were adequate.

The present data on unaffected occupancy of NMJ in the EOMs of transgenic mice at the advanced stages of the disease is in line with our findings in the EOMs of human ALS donors (22) - that the EOMs at the end-stage

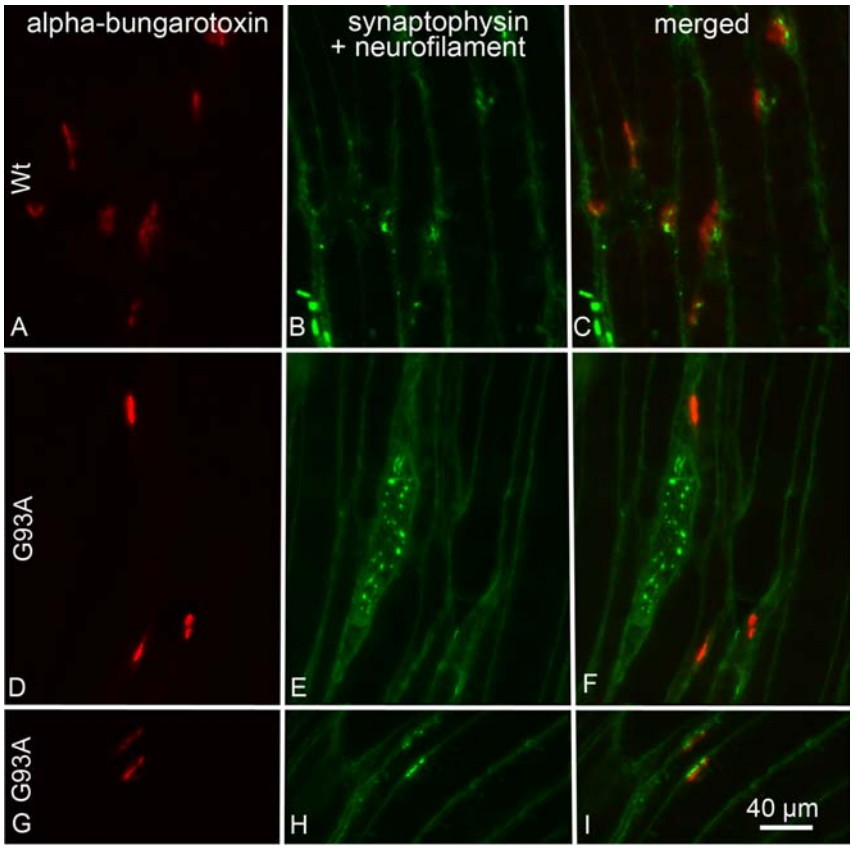

Figure 3. Neuromuscular junctions in limb muscles of Wt (81 days old, soleus A-C) and G93A (148 days old, gastrocnemius D-I) mice visualised with antibodies against synaptophysin + neurofilament (green) and with alpha-bungarotoxin (red) In A-C, normal, innervated NMJs can be seen. D-F depict three denervated NMJs from a G93A mouse where no overlap between synaptophysin (bright green) and alpha-bungarotoxin (red) can be seen. G-I display two examples of innervated NMJs in the same transgenic mouse, where adjacent staining of alpha-bungarotoxin and synaptophysin is clearly visible.

of ALS are less affected than the limb muscles. Recently, we also showed that the laminin alpha-4-chain, an important component of the basement membrane of NMJs, is well preserved at the NMJs of EOMs in ALS patients, as opposed to the NMJs of their limb muscles (33). We propose that the unique characteristics of the EOMs and their NMJs likely play a vital part in the slower progress of the disease in EOMs.

The EOMs, compared to other striated muscles, have fundamentally different properties and a distinct gene expression profile (34-36), features that make the EOM a separate muscle allotype. Among their distinct features are a glucose-based metabolism and possibly a better regenerative capacity than that of limb muscles (37). A study on rat NMJs has revealed that the RNA expression profile of NMJs in the EOMs differs greatly from that of NMJs in skeletal muscles (38). One of the differences found in the study was that NMJs in EOMs, in contrast to the NMJs of limb muscles, have a high expression of Slc24a2, an ion exchange membrane protein (39) that likely facilitates efflux of Ca^{2+} from the NMJ. Interestingly, a previous study has shown a noticeable defect in intracellular Ca^{2+} regulation at the NMJs of limb muscles in the G93A mouse (40).

The three components of the NMJ, the muscle fibre, the axonal nerve-end and the terminal Schwann cells, are all highly interdependent, and pathology and insults in one of these components will easily have devastating effects on the others (41, 42). The terminal Schwann cells play a vital role in the regulation at the NMJ, but do not seem to be the culprit behind ALS, as Schwann cell-specific expression of mutant SOD1 has no effect on the motor function or life expectancy of transgenic mice (43). Similarly, neuron-specific expression of SOD1 in a transgenic mouse model was not sufficient to trigger motor neuron degeneration in two studies (44, 45), whereas in another study the animals developed an ALS-like phenotype (46). In contrast, a growing body of evidence points out the muscle itself as a major player in the pathophysiology of ALS (3, 47). For example, it has been shown that muscle-restricted expression of either Wt or G93A hSOD1 results in an ALS-like phenotype with muscle denervation, muscular morphological abnormalities and motor neuron degeneration (29).

Trophic factors derived from the muscle also seem to play a role in slowing the disease progression, since muscle-specific up-regulation of insulin-like growth factor

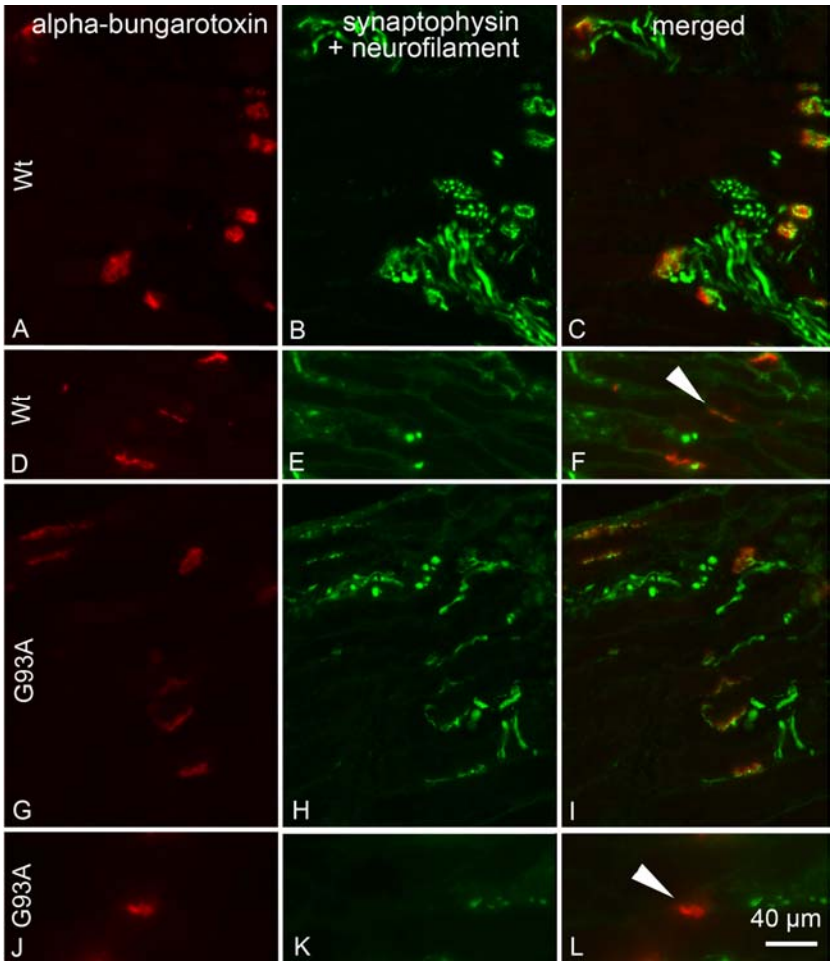

Figure 4. Neuromuscular junctions in extraocular muscles of Wt (44 days old, A-C; 110 days old, D-F) and G93A (119 days old, G-I;148 days old, J-L) mice visualised with antibodies against synaptophysin + neurofilament (green) and with alpha-bungarotoxin (red). In A-C, innervated NMJs and a nerve can be seen. D-F display an example of a rare, denervated NMJ in EOM of Wt (white arrowhead in F). G-I show a number of innervated NMJs in the EOM of a G93A mouse. Nerve fibres were abundant in both Wt and G93A muscle samples. J-L display a denervated NMJ (white arrowhead in L) in the EOM of a G93A mouse.

1 (IGF-1) decreases denervation, increases motor neuron survival and increases the lifespan of the G93A mouse (48). Notably, insulin-like growth factor binding protein 5, which increases the half-life of IGF-1 locally, has an expression level that is 7-fold higher in the NMJs of the EOMs of rat than in human limb muscles (38).

Taken together, these data indicate that the muscle likely plays an important part in the onset and progression of ALS, and that the inherent characteristics of muscle fibres influence this process. We propose that the relative sparing of the EOMs in ALS likely derives from the unique and intrinsic properties of these muscles and that they are a useful model to advance our understanding of ALS. Further studies are needed to elucidate how the specific differences between EOM and limb muscles may contribute to the superior resistance of the EOM to the underlying process of ALS. Hopefully, this may provide useful clues for the development of future therapeutical interventions with the intent to reduce the impact of the disease in skeletal muscles, thus prolonging survival.

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Abbreviations: ALS: amyotrophic lateral sclerosis; EOM: extraocular muscle; NMJ: neuromuscular junction; SOD1: superoxide dismutase 1; SALS: sporadic ALS; FALS: familial ALS; Wt: wild-type; IGF-1: insulin-like growth factor 1

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