Accumulation of STIM1 is associated with the degenerative muscle fibre phenotype in ALS and other neurogenic atrophies

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Aim: Upon denervation, skeletal muscle fibres initiate complex changes in gene expression. Many of these genes are involved in muscle fibre remodelling and atrophy. Amyotrophic lateral sclerosis (ALS) leads to progressive neurodegeneration and neurogenic muscular atrophy (NMA). Disturbed calcium homeostasis and misfolded protein aggregation both in motor neurones and muscle fibres are key elements of ALS pathogenesis that are mutually interdependent. Therefore, we hypothesized that the calcium sensor STIM1 might be abnormally modified and involved in muscle fibre degeneration in ALS and other types of NMA. Methods: We examined ALS and NMA patient biopsy and autopsy tissue and tissue from G93A SOD1 mice by immunohistochemistry and immunoblotting. Results: In normal human and mouse muscle STIM1 was found to be differentially expressed in muscle fibres of different types and to concentrate at neuromuscular junctions, compatible with its known role in calcium sensing. Denervated muscle fibres of sALS and NMA cases and SOD1 mice showed diffusely increased STIM1 immunoreactivity along with ubiquitinated material. In addition, distinct focal accumulations of STIM1 were observed in target structures within denervated fibres of sALS and other NMA as well as SOD1 mouse muscles. Large STIM1-immunoreactive structures were found in ALS-8 patient muscle harbouring the P56S mutation in the ER protein VAPB. Conclusion: These findings suggest that STIM1 is involved in several ways in the reaction of muscle fibres to denervation, probably reflecting alterations in calcium homeostasis in denervated muscle fibres.

Keywords: ALS, calcium homeostasis, denervation, neurogenic muscular atrophy, STIM1, tubular aggregate myopathy

Introduction

Lower motor neurones and the innervated muscle fibres form motor units. The motor neurones and their target muscle fibres within these units are structurally and functionally interdependent. For example, developing...
motor neurones die if deprived of their target muscles [1–3]. Conversely, muscle development is arrested in the absence of innervation [4]. Neurogenic muscular atrophy (NMA) [5] occurs if skeletal muscle fibres that have been innervated are denervated. NMA is a major feature of many disorders affecting the peripheral nervous system (PNS), including motor neurone diseases such as amyotrophic lateral sclerosis (ALS), spinal and spinobulbar muscular atrophies (SMAs and SBMAs) and sensorimotor peripheral neuropathies (PNPs) [6–11]. Recent evidence derived from experimental approaches suggests that in certain motor neurone diseases, muscle fibre alterations are not merely reactive, but causative for neuronal degeneration. For example, muscle knockdown of survival motor neuron (SMN) proteins leads to a much more pronounced phenotype than neuronal knockdown [12]. Similarly the SBMA mouse model expressing mutant androgen receptor (AR) containing a poly Q expansion develops a neuromuscular pathology with early muscle, but late spinal cord disease and relatively spared motor neurones very similar to SBMA in patients [13]. In fact, in most animal models of SBMA no detectable loss of motor neurone cell bodies occurs [13–15], suggesting that SBMA might be a lower motor neurone degeneration of muscle origin [16]. Similarly, the predominant pathogenic event in G93A SOD1 mouse model of ALS, determining the survival of the animals, is not motor neurone death but rather the loss of muscle to neurone connectivity; therapies targeting muscles and the neuromuscular axis as a whole yield global protection to motor neurones [17–19]. In addition, diminishing mutant (G37R) SOD1 protein selectively from motor neurones of G37R SOD1 transgenic mice could only delay onset and did not cure the disease [20]. Taken together, these data support the view that in many neuromuscular disorders degeneration of one component of the motor unit, either motor neurone or muscle fibre, leads to degeneration of the other [21].

Abnormal intraneuronal protein aggregates are a hallmark of ALS and other neurodegenerative diseases [22–25]. Protein aggregation, e.g. in so-called target structures, is also a major feature of denervated muscle fibres. Protein aggregation in both neurones and muscle is influenced by intracellular Ca\(^{2+}\) homeostasis. For example, pre-fibrillar ALS-associated protein aggregates have been shown to elevate cytosolic Ca\(^{2+}\) in neurones promoting the deleterious cascade [26,27]. Similarly, calcium deregulation in affected muscle fibres from ALS mice contributes to muscular atrophy [28]. In fact, Ca\(^{2+}\) is a major regulatory and signalling molecule in neurones as well as skeletal muscle, and any disruption of intracellular Ca\(^{2+}\) homeostasis such as elevated intracellular Ca\(^{2+}\) levels are known to trigger apoptosis and/or excessive phosphorylation of key proteins in ALS and SMA [26,29,30]. Conversely, the cellular Ca\(^{2+}\) dynamics need to be tightly regulated in order to guarantee the proper functioning of both motor neurones as well as muscle fibres [31,32]. However, it remains to be precisely established which abnormal inclusions and aggregation states are primarily responsible for the toxicity to the motor unit.

Stromal interaction molecule 1 (STIM1) is a single-pass transmembrane protein located in the sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER), where it functions as a Ca\(^{2+}\) sensor and activates Ca\(^{2+}\) entry by the Orai channels [33–35]. Although the function of STIM1 has been well characterized in non-excitable cells, the importance of STIM1 in skeletal muscle fibres has only recently been recognized [36]. Conditional deletion of STIM1 from mouse skeletal muscle leads to profound growth delay, reduced developmental myonuclear proliferation, and perinatal lethality due to disruption of tonic Ca\(^{2+}\) signalling in muscle fibres which attenuates downstream muscle growth signalling [37]. Human patients harbouring mutations in either STIM1 or Orai1 not only manifest profound immune deficiency but also display signs of a congenital skeletal myopathy and hypotonia [38]. Recently, STIM1 mutations have been identified as a genetic cause of tubular aggregate myopathy (TAM) and all mutations were found in the highly conserved intraluminal Ca\(^{2+}\) binding EF hands [39]. TAM cells expressing these mutants have significantly higher basal Ca\(^{2+}\) levels and a dysregulation of intracellular Ca\(^{2+}\) homeostasis [39]. Taken together, these findings provide clear evidence for the important role of STIM1 in the maintenance of Ca\(^{2+}\) homeostasis and in downstream regulation of skeletal muscle growth and function.

Denervation of muscle fibres leads to alterations in Ca\(^{2+}\) homeostasis, disassembly of myofibrillar and other structures as well as formation of protein aggregates. The mechanisms governing this standardized response are poorly understood. Therefore we asked whether the Ca\(^{2+}\) sensor STIM1 is altered in denervated muscle fibres and whether muscle fibres in biopsies of ALS patients are affected differentially compared to muscle fibres in other forms of denervation atrophy.
**Materials and methods**

**Human samples**

Human autopsy muscles samples were obtained from the Academic Medical Center (AMC) Brain Bank (Amsterdam, The Netherlands) following the guidelines of the local ethics committee. The muscles of these clinically confirmed sporadic and familial ALS patients as well as age-matched controls had been removed within 6–12 h after death. All ALS patients had suffered from clinical signs and symptoms of lower and upper motor neurone disease with the eventual involvement of brain stem motor nuclei. Importantly, none of these patients had cognitive impairment or dementia. Age-matched control patients did not show any neuropathological abnormalities. Human muscle biopsies from clinically confirmed sporadic and familial ALS patients, from patients with clinically and histologically confirmed NMAs due to sensorimotor neuropathies including microangiopathic/diabetic neuropathy and neuritis as well as age-matched control muscle biopsies were obtained from the archives of the Institute of Neuropathology, RWTH Aachen University Hospital, following the guidelines of the Ethics Committee of RWTH Aachen University Hospital. Samples of control and diseased muscles were processed in parallel. In total, muscle samples from 14 ALS and 6 control autopsy cases and muscle biopsies from 11 ALS patients, 13 NMA patients and 6 controls were examined by histology and immunohistochemistry. Seven ALS patients, 7 NMA patient and 3 control muscle samples were examined by immunoblotting.

**Experimental animals**

All procedures were approved by the RWTH Aachen University Hospital Institutional Animal Care and Use Committee. ALS mice expressing high copy numbers of human mutant G93A-SOD1 \[40\] were obtained from Jackson Laboratories [Bar Harbor, ME, USA; strain designation B6SJL-Tg (SOD1 G93A) 1Gur/J]. Homozygous G93A SOD1 mice were maintained by mating G93A males with B6SJL/J hybrid females. Transgenic litters were genotyped using PCR for human SOD1 from tail tissue [40]. Severely affected 18 week old male mice and their corresponding control littersmates were used for all experiments \(n = 3\) each. To ensure optimal quality of SOD1 mutant and control mouse muscles for immunohistochemical investigation, tissues were either fixed by transcardial perfusion with ice-cold 2% paraformaldehyde (PFA) containing 2% picric acid (pH 7.4) or immediately frozen in liquid nitrogen, see also [29].

**Reagents**

Fluorescent nucleic acid stain Hoechst 33258 and vectashield mounting medium with DAPI were from Molecular Probes (Life Technologies GmbH, Munich, Germany) and Vector Laboratories (Peterborough, UK). MG-132, thapsigargin, bafilomycin and rapamycin were purchased from Sigma Aldrich (St Louis, USA). All antibodies and their dilutions are listed in Table S1.

**Immunohistochemistry, histochemistry**

Transverse paraffin or cryostat sections of human and mouse muscles were placed on silane-coated slides, de-waxed, rehydrated and heated in citrate buffer for antigen retrieval (for paraffin sections) while the cryostat sections were directly processed for antigen retrieval after bringing the slides to room temperature. Processed sections were incubated with primary antibodies for 1 h at room temperature. Appropriate HRP secondary antibodies were used (1:200, Vector Laboratories, USA) for 1 h, followed by DAB visualization (DAKO, Denmark). For immunofluorescence secondary antibodies conjugated with Alexa fluorophore (Invitrogen; Life Technologies GmbH) were used. Stainings were visualized using a Zeiss LSM 700 confocal microscope (Zeiss, Göttingen, Germany). The resulting confocal images were processed using the Zeiss LSM software and Adobe Photoshop CS5.

Standard histological and histochemical stainings including H&E, ATPases, PAS, Oil red O, myoadenylate deaminase, modified Gomori trichrome, non-specific esterase and NADH were performed as described [41]. The histological, histochemical and DAB immunohistochemical sections were photographed using an Axioplan microscope (Zeiss) with an Axio Cam HR camera.

**Electron microscopy**

Transmission electron microscopy of muscle tissue fixed in 6% phosphate-buffered glutaraldehyde was performed using standard protocols as described [42] using a Philips EM 400 T electron microscope equipped with a Morada digital camera.
Western blot analysis

Frozen muscle tissues were homogenized in lysis buffer (0.5% Triton X-100 in PBS, 0.5 mM PMSF and complete protease inhibitor mixture (Roche Applied Sciences, Switzerland). After incubation on ice for 30 min, lysates were briefly sonicated. Clear lysates were obtained after brief centrifugation for 5 min at 6000 rpm. Protein concentrations were determined using the BCA method (Molecular Probes). Equal amounts of protein were boiled for 5 min in 2× SDS-sample buffer and subjected to 10 or 12% SDS-PAGE prior to transfer to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% skimmed milk in 0.05% Tween 20/Tris-buffered saline (TBS-T) for 30 min prior to incubation with primary antibodies at a dilution of 1:1000. The membranes were incubated overnight at 4°C, then washed 3 times in TBS-T and incubated for 1 h in appropriate horseradish peroxidase-conjugated secondary antibody (1:10 000, Thermo Scientific). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences); densitometric quantification of the band intensity normalized to tubulin levels was performed using Adobe Photoshop CS5.

Results

STIM1 is differentially expressed in muscle fibre types of innervated muscle and is concentrated at neuromuscular junctions

Innervated human muscle fibres showed STIM1 immunoreactivity associated with sarcomeric bands (Figure 1A); STIM1 partially co-localized with ryanodine receptor (RyR) and SERCA1 (Figure 1A,B). STIM1 labelling was consistently more intense in human type II muscle fibres compared to human type I fibres (Figure 1C). In innervated mouse muscle, type II fibres also showed a higher staining intensity than type I fibres (Figure 1D, upper and middle panel). In line with these results we observed partial co-localization of STIM1 immunoreactivity with SERCA1 staining (Figure 1D, lower panel); SERCA1 is predominantly expressed in type II fibres [43]. We were especially interested in the expression profile of STIM1 at the neuromuscular junction (NMJ), because Ca2+ signalling is prominent at these specialized structures. STIM1 protein was found to concentrate at postsynaptic sites of NMJ of normal-sized fibres in innervated and denervated muscles of both human patients and human controls and of normal mice (Figure 1E,F; Figure 2B). The presence of NMJs was confirmed by staining for the presynaptic marker synaptophysin (Figure 1E) and by esterase histochemistry (Figure 1F). We next analysed the expression of STIM1 in mouse gastrocnemius and soleus muscles by Western blotting. Soleus muscle of C57BL6 mice is composed predominantly of type I and IIA fibres whereas gastrocnemius muscle predominantly contains IIB and IIDB fibres [44,45]. We observed significantly higher levels of STIM1 protein in mouse soleus muscles compared to gastrocnemius muscles (Figure 1G,H), which suggests that mouse type IIA muscle fibres contain higher levels of STIM1 than type IIB fibres.

STIM1 accumulates in denervated muscle fibres in NMA

Immunohistochemical staining revealed a consistent accumulation of STIM1 in partially (diameter 20–40 μm) and completely (diameter <20 μm) atrophic muscle fibres (Figure 2A) in NMA. These fibres were often arranged in groups and found to be esterase-positive (Figure 2A). The presence of ubiquitinated inclusions along with STIM1 accumulation confirmed the activation of protein degradation in such denervated muscle fibres (Fig. S2A). STIM1 immunoreactivity was not precisely co-localized with the ubiquitinated structures (Fig. S2A). There was a consistent intense staining of the centre of many targets and of ring-like structures around targets (Figure 2B, a,b,f). Muscle fibres displaying secondary myopathic features such as fragmentation and non-subsarcolemmal myonuclei showed intense dispersed STIM1 immunoreactivity (Figure 2B, c,e). Immunohistochemical staining of the targets demonstrated the involvement and accumulation of the autophagic substrates p62 and Lamp1 (Figure 2C). In contrast to normal human muscle, STIM1 immunoreactivity was not limited to fast (Type II) fibres but was also observed in partially atrophic slow fibres (Type I) (Fig. S2B). Electron microscopy revealed widening of presumably SR-derived tubular structures, triad proliferations and signs of autophagy especially in target structures and in the perinuclear region (Figure 2D, Fig. S2C). In the centre of targets (zone 1) and in the perinuclear region, the irregular, probably SR-derived tubular structures were often not associated with T-tubules. Western blot analysis confirmed the increased levels of both isoforms (80 and
Figure 1. STIM1 in normal human muscle fibres. (A and B) Human muscle biopsy paraffin sections (n = 6 controls) showing a pattern of STIM1 immunofluorescence staining associated with sarcomeric bands co-localized with RyR1 (upper panel) and with SERCA1 (lower panel) in normal-sized, innervated fibres. Scale bar = 30 μm. (B) STIM1 and RyR1 (green) co-localization at a higher magnification (left panel; scale bar = 15 μm). Immunoreactivity for STIM1 alone using diaminobezidine labelling (right panel; scale bar = 30 μm). (C) Differential staining of slow twitch (type I, indicated as I) and fast twitch (type II, indicated as II) human muscle fibres. There is prominent STIM1 staining in type II fibres in normal human muscle. Cryostat sections; scale bar = 100 μm. (D) Normal mouse muscle. Upper panel: STIM1 staining is more intense in type II compared to type I fibres (cryostat sections, ATPase staining; scale bar = 100 μm). This pattern is confirmed by MHC (middle panel) and SERCA1 (lower panel) staining of paraffin sections. Scale bars = 100 μm. (E) Fluorescence immunostaining showing the strong STIM1 labelling at human NMJs co-stained for the presynaptic marker synaptophysin. Paraffin section; scale bar = 80 μm. (F) Prominent STIM1 staining at NMJs in normal mouse muscle; esterase staining confirming the presence of NMJ (lower panel). Representative section for n = 3 mice examined. Frozen section; scale bar = 100 μm. (G) Western blot analysis of STIM1 protein demonstrating lower expression levels in gastrocnemius vs. soleus muscles in normal mice (n = 3). (H) Quantitative densitometric analysis of the findings depicted in (G) (G: gastrocnemius, S: soleus muscles). *P < 0.05.
28 kDa) of STIM1 protein in human NMA muscle and the active involvement of autophagy as shown by increased levels of the autophagy markers p62 and LC3II (Figure 2E,F).

**STIM1 accumulates in the denervated muscle fibres in ALS**

Immunohistochemical analysis of ALS patient muscle samples (both frozen and paraffin-embedded) revealed a considerable accumulation of STIM1 in partially (diameter 20–40 μm) and completely (diameter <20 μm) atrophic fibres whereas the non-atrophic muscle fibres were barely stained (Figure 3A,B). Again, ubiquitinated inclusions were seen along with, but not completely co-localized with STIM1 accumulations, confirming the activation of protein degradation (Fig. S3A). As in the NMA cases, STIM1 accumulations were prominent in the centre and around targets and were also present as globular inclusions in fibres undergoing secondary myopathic changes (Figure 3B). EM analysis of such fibres also revealed autophagy involvement similar to the NMA cases (Fig. S3B, a,b,c) with focal proliferation and swelling of the SR and accumulation of other organelles (Fig. S3B, d). Similar to NMA STIM1 immunoreactivity was not limited to fast (Type II) fibres, but was also observed in partially atrophic slow fibres (Type I) (Figure 3C). Western blot analysis of frozen muscle tissue both from autopsy and biopsy samples revealed a significant accumulation of STIM1 protein in ALS patient muscles as compared to controls (Figure 3D,E,G), confirming the immunohistochemical staining results. As in the NMA group, staining intensity of partially atrophic and atrophic fibres correlated with the degree of fibre atrophy. The ALS cases showed a higher number of partially and completely atrophic, esterase-positive muscle fibres and thus also a higher density of STIM-immunoreactive fibres. However, the intensity of the staining of the individual atrophic and partially atrophic fibres did not differ between the ALS and NMA groups (Figures 2, 3). There was no significant difference in the average STIM1 protein levels detected by immunoblotting in ALS vs. NMA muscles (Figure 3F,H).

**STIM1 accumulates in denervated muscle fibres in an ALS-8 patient muscle biopsy**

Vesicle associated membrane protein B (VAPB) is an ER-associated protein, as is STIM1. Therefore, we were interested in alterations in STIM1 protein distribution in ALS-8, which is due to VAPB mutation. We therefore retrieved the archival paraffin-embedded muscle biopsy of a recently identified ALS-8 patient who carries the P56S VAPB mutation [46,47]. We found a prominent diffuse increase of STIM1 immunoreactivity in partially atrophic and atrophic muscle fibres, similar to the other ALS and NMA cases (Figure 4A,B). In addition, we found peculiar large focal accumulations of STIM1 immunoreactivity in denervated muscle fibres of this case (Figure 4B, enlarged). Consistent with the previous results of NMA and sporadic ALS cases, p62 accumulation in the atrophic fibres of the ALS 8 case also suggested the active involvement of autophagic degradation in these fibres (Figure 4C).

**STIM1 accumulates in denervated muscle of the G93A SOD1 mouse model of ALS**

Having shown that STIM1 accumulates in muscle fibres of NMA and ALS patients, we next wanted to confirm our findings in a mouse model of familial ALS. We used the well characterized G93A SOD1 transgenic mouse model [40]. STIM1 immunohistochemistry showed co-localization of STIM1 with SERCA1 and RyR1 in normal muscle fibres (Figure 5A). STIM1 was found to accumulate in atrophic and partially atrophic mouse muscle fibres as well as in fibres undergoing secondary myopathic changes analogous to the findings in human ALS and NMA muscle (Figure 5B). As in human ALS we observed STIM1 accumulation in both fast and slow atrophic and partially atrophic muscle fibres in paraffin and frozen sections (Figure 5F,G). Furthermore STIM1 staining was associated with RyR1 staining in partially atrophic fibres (Figure 5F, lower panel). Immunoblot analysis confirmed the accumulation of both STIM1 isoforms in the affected muscles (Figure 5C,D) fibres. RT-PCRs were performed with mouse gastrocnemius muscles to study the expression of STIM1 at the mRNA level. STIM1 mRNA expression was significantly reduced, indicating that the increased levels of STIM1 protein were due to decreased degradation (Figure 5E). In parallel we observed a prominent increase in the autophagy marker LC3II (Figure 5C,D). Taken together, these findings support the notion that the autophagic machinery is activated and probably even overloaded in muscle fibres undergoing neurogenic atrophy, leading to the accumulation of substrates. In addition, STIM1 degradation might be actively and selectively spared from degradation in the denervated muscle fibres, possibly...
because of essential functions in these stressed fibres (see Discussion). In line with the assumption of a special role for STIM1 in this context, only slight increases in SERCA1 and other triad markers such as calsequestrin1 and Orai1 in SOD1 mice muscles were detected (Figure 5C,D). This suggests that the increase in STIM1 is not just due to a general (relative) increase in SR proteins in the denervated muscle fibres. Finally, STIM1 protein levels in muscle cell lines (RCMH) increased significantly after treatment with the autophagy inhibitor bafilomycin-A and the proteasome inhibitor MG132, confirming that inhibition of these degradation pathways leads to increased STIM1 levels (Fig. S4).

Discussion

STIM1 expression in normal muscle

In longitudinal sections of both mouse and human muscle fibres, STIM1 immunoreactivity was associated with sarcomeric bands (Figure 1A,B), which is consistent with the known location of STIM1 in the SR [48]. In normal fibres of both species, STIM1 was selectively accumulated in the postsynaptic sarcoplasm of NMJs. These observations are compatible with the known function of STIM1 as a Ca\(^{2+}\) sensor in the ER and the finding that the junctional sarcoplasm contains a dense endoplasmic reticulum [49]. Interestingly, in normal, innervated human muscle, type II (fast) muscle fibres were more prominently stained than type I (slow) fibres. Similarly, STIM1 is found in type II fibres in normal mice. In mice, the division of type II fibre population in certain subtypes differs from human (and rat) muscles [44,45,50]. Our immunoblot results are consistent with a selective expression of STIM1 in C57/Bl-6 mouse type IIA soleus muscle fibres (Figure 1G). However, this hypothesis requires conformation in future studies using elaborate enzyme histochemical fibre typing of several different muscles.

Accumulation of STIM1 in denervated muscle fibres

Denervation of adult muscle alters the expression levels of numerous structural and other proteins within days. The early phase is characterized by increased expression of transcription factors including immediate early genes such as c-jun and c-fos as well as of myogenic differentiation factors, including myoD, myogenin and MRF4 [51,52]. Cell surface proteins such as neural cell adhesion molecule (NCAM) and nicotinic acetylcholine receptors (nAChR) normally selectively expressed at the NMJ of adult muscle fibres are re-expressed in extra-synaptic parts of muscle fibres. Moreover, neurotrophic factors such as glial cell line–derived neurotrophic factor (GDNF) and ciliary neurotrophic factor α (CNTFR-α) are expressed at increased levels; they are thought to attract regenerating nerve fibres and/or to promote sprouting from terminal axons that are still intact [53,54]. Atrophy of muscle fibres that are not re-innervated is accompanied by decreased expression of structural proteins and increased expression of proteins executing muscle fibre breakdown [55,56].

In the present study, we found that STIM1 protein accumulates in denervated human and mouse muscle fibres, suggesting that the increase of STIM1 is part of the dynamic response of skeletal muscle fibres to denervation. STIM1 immunoreactivity is increased in partially atrophic and atrophic muscle fibres, in degenerating fibres undergoing necrosis and in target structures. This pattern is again compatible with the known role of STIM1 in Ca\(^{2+}\)
Figure 3. STIM1 accumulation in the denervated fibres in human ALS. (A) Prominent accumulation of STIM1 immunoreactivity in the denervated, atrophic muscle fibres. They are grouped, angular or flattened and often esterase-positive. Section from a representative case from the group of 13 ALS cases available for cryostat sectioning. Cryostat sections; scale bar = 150 μm. (B) Intense STIM1 staining of partially atrophic and atrophic muscle fibres in ALS (upper panel), target fibres (upper and middle panel) and of a fibre showing secondary myopathic alterations (lower panel). Paraffin sections; scale bars in the upper panel = 150 μm, in the middle and lower panel = 40 μm. (C) STIM1 immunoreactivity is increased in both fast and slow denervated muscle fibres. Paraffin sections; scale bar = 50 μm. (D, E, G) Western blot analysis of frozen tissue from sALS autopsy (n = 8) and sALS biopsy (n = 7) muscle specimens showing the accumulation of STIM1 in these muscles as compared to controls (n = 5 autopsies and 3 biopsies). *P < 0.05. (F, H) Western blot analysis to compare levels of STIM1 in ALS (n = 5) vs. NMA (n = 5) muscle homogenates from frozen human muscle biopsy specimens shows that levels of accumulation are similar in NMA and ALS muscles. #: not significant.
Figure 4. STIM1 accumulation in the denervated fibres in a case of ALS-8. (A) Prominent accumulation of STIM1 in atrophic, denervated fibres of a case of ALS-8. Paraffin section; scale bars = 120 μm. (B) Intense immunofluorescence staining of STIM1 in atrophic muscle fibres in ALS-8. Note the globular pattern of STIM1 labelling. Paraffin section; scale bars = 25 μm. (C) Accumulation of p62 in the denervated muscle fibres in ALS-8. Paraffin section; scale bars = 30 μm.
Figure 5. STIM1 accumulation in the denervated fibres in SOD1 tg mice. (A) Mouse gastrocnemius muscle sections (n = 3 controls) showing a pattern of STIM1 immunofluorescence staining associated with sarcomeric bands co-localized with SERCA1 (left panel) and RyR1 (right panel) within normal-sized, innervated fibres. Scale bar left panel = 50 μm, scale bar right panel = 25 μm. (B) Accumulation of STIM1 in the denervated, partially atrophic and atrophic muscle fibres in an SOD1 mouse muscle. Representative consecutive cryostat sections of muscle specimens from n = 3 mice; scale bar = 60 μm. (C) Western blot analysis of frozen tissue demonstrating that STIM1 as well as LC3 protein levels in gastrocnemius muscles of 4 SOD1 mice show a prominent increase compared to 3 wt littermate controls. Note that protein levels of the triad markers SERCA1, calsequestrin and Orai1 are only slightly, if at all, increased. (D) Quantitative densitometric analysis of the findings depicted in (C). *P < 0.05; # = not significant. (E) RT PCR analysis showing reduced STIM1 mRNA expression in 18 week-old SOD1 mouse gastrocnemius muscle (n = 3 mice each). *P < 0.05. (F) Comparison of STIM1 immunoreactivity in denervated slow and fast muscle fibres and in relation to RyR1 immunoreactivity. Paraffin sections; scale bar = 60 μm. (G) Comparison of STIM1 staining in denervated fast and slow muscle fibres. Fast (left panel), slow (right panel). Frozen sections; scale bars = 60 μm.
metabolism, which is up-regulated in the course of all stress responses and remodelling processes [11]. More specifically, alterations in Ca\(^{2+}\) homeostasis have been found to contribute to muscle fibre atrophy after denervation [28,30,57,58]. Notably, the increased STIM1 protein levels in denervated muscle fibres are not caused by an elevation of mRNA levels, but are due to decreased protein degradation. This notion is supported by the observation that RCMH skeletal muscle cells treated with the degradation inhibitors bafilomycin and thapsigargin show increased STIM1 protein levels (Fig. S4).

STIM1 immunoreactivity was increased in individual denervated fibres of NMA patients in cases of PNP following the same pattern as found in most human and all mouse ALS muscles, suggesting that STIM1 up-regulation is caused by denervation and is not due to a primary muscle fibre process that is different between PNP and most cases of ALS. However, in the muscle biopsy obtained from an ALS8 patient [46,47], we observed large globular STIM1-immunoreactive structures in the perinuclear sarcoplasm of partially atrophic and atrophic muscle fibres. This finding suggests that the defect in the ER-associated VAPB gene causing ALS-8 might primarily affect skeletal muscle fibres.

**STIM1 selectively accumulates in targets**

Targets are focal denervation-specific muscle fibre alterations [59]. They are typically composed of three concentric zones: a more or less dense central zone (zone 1), a loosely structured intermediate zone (zone 2) with a diminution of fibrillar structures, and a light microscopically normal or slightly altered peripheral zone (zone 3) [60]. We consistently observed STIM1 accumulation in the centre (zone 1) and at the periphery (zone 3) of targets (Figure 2B). This peculiar pattern is easily explained in case of zone 3, because the SR is known to be dilated at the rim of targets [60]. However, SR has been described to be absent from zone 1 [60]. On the other hand, degradation products which form at the target centre have been described to acquire a single limiting membrane, move to the fibre surface, and are extruded [61]. In our own electron microscopic studies we found variable degrees of vacuolization indicating SR widening and autophagy over the entire profile of targets including zone 1 (Figure 2D; Figs S2,S3), confirming the notion that autophagy is involved in muscle fibre atrophy [62]. It is conceivable that the accumulation of STIM1 in zone 1 actually reflects the segregation of SR compartments and that STIM1 accumulates in targets to serve its known functions in Ca\(^{2+}\) sensing and homeostasis. Calcium homeostasis tightly regulates autophagy [63,64] and STIM1 might thus be involved in the regulation of autophagy in targets.

**Conclusions**

Taken together, our findings suggest that the Ca\(^{2+}\) sensor STIM1 is involved in several ways in the reaction of muscle fibres to denervation, probably reflecting alterations in Ca\(^{2+}\) homeostasis and protein degradation in denervated muscle fibres. Thus, STIM1 and related proteins are potential targets of therapeutic approaches to inhibit muscle fibre atrophy after denervation.

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**Conflict of interest statement**

No conflict of interest.

**Author contributions**

A.G., C.M.J. and J.W. raised the hypothesis and designed the experiments. Experimental work was performed by A.G., C.M.J., A.C., E.B., T.V., A.D., and K.I. M.G. and S.W. provided ALS and NMA cases. The ALS and NMA cases were examined by D.T., S.N. and J.W. The SOD1 transgenic mice were maintained and prepared by S.J. and C.B. The manuscript was written by A.G., C.M.J. and J.W. J.W. supervised the entire project. All authors discussed results and commented on the manuscript.
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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Negative controls of atrophic and normal muscle fibres. Muscles sections immunostained without primary antibody showing absence of immunolabelling. (A) Human ALS, (B) human NMA, (C) 18 week old wt mouse and (D) 18 week old SOD1 mouse. Representative paraffin sections (n ≥ 3 for each group); scale bars in (A) and (B) = 70 μm, in (C) and (D) = 120 μm.

**Figure S2.** Findings in denervated fibres in NMA muscle biopsies. (A) Prominent accumulation of both STIM1 and ubiquitin in the denervated fibres detected by immunofluorescence. Representative paraffin sections (n = 11 cases examined); scale bar = 50 μm. (B) STIM1 immunoreactivity is increased in both fast and slow denervated and re-innervated, target-containing muscle fibres. Paraffin sections; scale bar = 50 μm. (C) EM of the margin of a target structure (zone 3) showing the typical Z-band aberrations and focal widening of the sarcoplasmic reticulum (arrows). Scale bar = 350 nm.

**Figure S3.** STIM1 accumulation in the denervated muscle fibres in human ALS. (A) Accumulation of both STIM1 and ubiquitin in the denervated fibres. Representative paraffin sections (n = 13 cases examined); scale bar = 60 μm. (B) EM showing variable degrees of ER proliferation and widening (arrows) and of vacuolization indicating autophagy (asterisk) in denervated muscle fibres. Scale bar in (a) = 2 μm, in (b) = 0.8 μm, in (c) = 2 μm, in (d) = 0.4 μm, in (e) = 1 μm.

**Figure S4.** Accumulation of STIM in RCMH cells. RCMH cells were treated with increasing concentrations of, proteasomal inhibitor MG132 (0.5, 1, 1.5 & 2 μM), ER stressors thapsigargin (0.5, 1, 1.5 & 2 μM) and autophagy inhibitor bafilomycin A (10, 50, 100, 200 nM) for 6 hrs. Cells were collected, lysed and prepared for Western blot analysis using antibodies as indicated.

**Table S1.** List of the antibodies used.

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