TITLE:

What skeletal muscle has to say in ALS: implications for therapy

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Nomenclature for drug/molecular targets conforms to BJP's Concise Guide to Pharmacology (Alexander et al., 2017).

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an adult onset disorder characterised by progressive neuromuscular junction (NMJ) dismantling and degeneration of motor neurons leading to atrophy and paralysis of voluntary muscles responsible for motion and breathing. Except for a minority of patients harbouring genetic mutations, the origin of most ALS cases remains elusive. Peripheral tissues, and particularly skeletal muscle, have lately demonstrated an active contribution to disease pathology attracting a growing interest for these tissues as therapeutic targets in ALS. In this sense molecular mechanisms essential for cell and tissue homeostasis have been shown to be de-regulated in the disease. These include muscle metabolism and mitochondrial activity, RNA processing, tissue-resident stem cell function responsible for muscle regeneration, and proteostasis that regulates muscle mass in adulthood.

This review aims to compile scientific evidence that demonstrates the role of skeletal muscle in ALS pathology and serve as reference for development of novel therapeutic strategies targeting this tissue to delay disease onset and progression.

NON-STANDARD ABBREVIATIONS

Neuromuscular junction (NMJ), mutant SOD1 (mSOD1), mitochondrial DNA (mtDNA), dipeptide repeat (DPR), RNA binding proteins (RBPs), extensor digitorum longus (EDL), slow-twitch soleus (SOL).

KEYWORDS

Neurodegeneration/neuroprotection, intercellular signalling, cellular repair mechanisms, Amyotrophic lateral sclerosis, regeneration, proteostasis, RNA metabolism.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neuromuscular disorder of unknown etiology. While the vast majority of the ALS cases are sporadic, a minority (5-10%) are caused by inherited familiar gene alterations (Byrne et al., 2011). Mutations in more than 25 ALS-linked genes have been described, with those encoding cytoplasmic superoxide dismutase 1 (Sod1) and guanine nucleotide exchange factor C9orf72 being most frequently affected (Al-Chalabi & Hardiman, 2013; Nguyen, Van Broeckhoven, & van der Zee, 2018). Disease onset is secondary to upper and lower motor neuron degeneration, appears around 55-60 years, and is accompanied with cramping and twitching, hyperreflexia, spasticity, severe muscle weakness and atrophy in limbs and trunk (spinal form) or dysarthria and dysphagia (bulbar form). As disease progresses, patients are also at risk of choking on food and aspiration pneumonia. ALS is a fast-decline disorder that progresses to paralysis of voluntary muscles including diaphragm and intercostals responsible for breathing, with an average life expectancy of 3-5 years after diagnosis.

Unlike other neuromuscular disorders such as spinal and bulbar muscular atrophy (SBMA), where toxicity associated to androgen receptor mutation in skeletal muscle is widely accepted to contribute to disease pathogenesis and progression and this tissue is a target for drug development (Manzano et al., 2018), controversy exists on the actual etiology of denervation, muscle atrophy and weakness in ALS. Whether it is solely a consequence of motor neuron degeneration or there is also a contribution from pathogenic mechanisms arising from skeletal muscle or other peripheral tissues is still under debate. Similarities in skeletal muscle between ALS and SBMA include incomplete myoblast differentiation *in vitro* (Malena et al., 2013), histological signs of neurogenic together with primary myogenic defects (i.e. presence of angulated and grouped fibers and centrally nucleated and necrotic myofibers) and glycolytic-to-oxidative metabolic fiber-type switching (Rocchi et al., 2016).

However, mutant SOD1 (mSOD1) gene excision or inhibition of its transcription or protein expression in skeletal muscle does not preserve grip strength, nor affects survival or disease onset in the SOD1-G93A mouse model of ALS (Miller et al., 2006). On the contrary, latter studies have shown that restricted expression of mSOD1 in skeletal muscle of mice at similar levels as in the SOD1-G93A mouse model (where a human SOD1 enzyme carrying a G93 to A mutation is ubiquitously expressed), causes muscle atrophy and reduces tetanic and specific force, displaying neuromuscular junction abnormalities and a motor neuron distal axonopathy (G. Dobrowolny et al., 2008) (Wong & Martin, 2010). These results highlight the importance of unravelling the temporal dynamics and mechanisms involved in skeletal muscle phenotype in ALS.

This review discusses the current understanding about the role of skeletal muscle in ALS, focussing on the pathogenic molecular pathways and the potential therapeutic approaches targeting this tissue (Figure 1). This information is aimed to be useful in the search for novel mechanisms, a better management of ALS patients and the design of novel drugs and less invasive delivery routes to tackle this disorder.

NEUROMUSCULAR JUNCTION AND THE DYING BACK PHENOMENON

Motor neuron nerve terminals and muscle fibers in vertebrates communicate through highly specialized cholinergic synapses, the neuromuscular junctions (NMJ). These presynaptic terminals receive action potentials and trigger the release of neurotransmitter acetylcholine that subsequently activates nicotinic acetylcholine receptors in postsynaptic muscle fibers enabling muscle contraction. The NMJ also contains other essential synapse-associated cells, including perisynaptic Schwann cells involved in the development, maintenance and regeneration of NMJ function through trophic cues (reviewed in (Lepore, Casola, Dobrowolny, & Musaro, 2019). Increasing evidence from ALS animal models such as those of various mSOD1, mutant fused in sarcoma (FUS) and nuclear localization -defective TAR DNA-binding protein 43 (TDP-43) indicates that NMJ defects take place in the early stage of the disease in ALS, the fast-type (fast-fatigable) neuromuscular synapses being the most vulnerable (Acevedo-Arozena et al., 2011; Fischer et al., 2004; Frey et al., 2000; Hadzipasic et al., 2014; Hegedus, Putman, & Gordon, 2007; Pun, Santos, Saxena, Xu, & Caroni, 2006; Sharma et al., 2016; Spiller et al., 2016). From the NMJ, the pathology proceeds along the axons to the motor neuron cell body with a "dying-back" pattern, leading to motor neuron death as a relatively late event. These observations are consistent with reports that expression of mSOD1 in motor neurons alone is not sufficient to cause ALS-like symptoms in mice (Lino, Schneider, & Caroni, 2002; Pramatarova, Laganiere, Roussel, Brisebois, & Rouleau, 2001; Yamanaka et al., 2008), although some studies disagree with this (Jaarsma, Teuling, Haasdijk, De Zeeuw, & Hoogenraad, 2008; L. Wang et al., 2008). However, preventing motor neuron apoptosis does not ultimately protect from NMJ denervation (Gould et al., 2006). To what extent and in which specific conditions the NMJ stability is determined by signals originating from one or another tissue or cell type remains under investigation. The contribution to motor neuron degeneration by non-neural cells including glial cells (Boillee, Yamanaka, et al., 2006; Carrasco, Bichler, Seburn, & Pinter, 2010; Clement et al., 2003; Ferraiuolo et al., 2016; Yamanaka et al., 2008) and skeletal muscle (Lepore et al., 2019; Wong & Martin, 2010) is accumulating, and suggests that alterations in multiple cell types act synergistically to exacerbate the disease (Boillee, Vande Velde, & Cleveland, 2006).

Although direct investigation on sequential molecular events leading to death of motor neurons is somewhat limited in ALS patients, it is known that synaptic protein composition is disturbed in NMJ associated with severely affected muscles of ALS patients, whereas those preserved (such as extraocular muscles) are only mildly affected (Liu, Brannstrom, Andersen, & Pedrosa-Domellof, 2013). This suggests that molecules involved in NMJ function and stability could serve as early biomarkers for the distal axonopathies, and that interventions that aim to preserve NMJ could serve for therapeutic purposes. One of such factors is RTN4 (Nogo-A), a neurite outgrowth inhibitor overexpressed in the skeletal muscle of ALS patients (P. F. Pradat et al., 2007), where its levels also correlate with disease severity (Jokic et al., 2005) and endplate denervation (Bruneteau et al., 2015). In mSOD1 mice RTN4 promotes denervation (Jokic et al., 2006) and antibody treatment against this protein ameliorates the disease progression and, therefore, may serve as a promising therapeutic target (Bros-Facer et al., 2014). However, it is not yet mechanistically clear how RTN4

promotes denervation in ALS as its expression has been described to be limited in slow muscle fibers (Jokic 2005) which in ALS are generally regarded as the most resistant ones. Additionally, some studies have described increased immunoreactivity of RTN4 in other muscle pathologies (Wojcik, Engel, & Askanas, 2006) which may limit its use as a highly specific ALS biomarker.

IMPAIRMENT OF MUSCLE MITOCHONDRIAL ACTIVITY AND METABOLISM

Although much less studied than for motor neurons, mitochondrial abnormalities have been also described in skeletal muscle from ALS patients. Those include ultrastructural changes such as swelling, paracrystalline inclusions, disruption of the cristae and dilution of the matrix (Napoli et al., 2011). These aberrant mitochondria have been located frequently in aggregates adjacent to subsarcolemma (Chung & Suh, 2002). Manganese superoxide dismutase (Mn-SOD) is located in mitochondria being responsible for detoxification of the superoxide anion radical ($O_2^{-\bullet}$), the major ROS generated in this organelle into molecular oxygen (O2) and hydrogen peroxide (H2O2) (Skulachev, 1996), representing a cellular defence against reactive oxidative species (ROS). A 40% reduction in Mn-SOD has been found in membrane fractions obtained skeletal muscle homogenates from sporadic ALS patient (Vielhaber et al., 2000). In agreement, accumulation of ROS and oxidative stress have been reported in skeletal muscle from ALS patients and SOD1-G93A mice (Yajuan Xiao et al., 2018). These mice also showed an increase in Cyclophilin D (CypD) expression which promotes the opening of the mitochondrial permeability transition pore (mPTP), increasing mitochondria membrane depolarization and the further generation of ROS species (Yajuan Xiao et al., 2018; Zorov, Juhaszova, & Sollott, 2014).

In turn, excessive ROS production is reported to exacerbate mitochondrial impairment by damaging mitochondrial DNA (mtDNA). Multiple mtDNA deletions, depletion of intact mtDNA and decreased activity of the mitochondrial encoded enzymes responsible for respiratory chain (NADH:cytochrome *c and* NADH:Coenzyme Q oxidoreductases and Cyclooxygenase *(*COX)) have been described in skeletal muscle from familial ALS patients (Artuso et al., 2013; Crugnola et al., 2010; Vielhaber et al., 2000; Wiedemann et al., 1998), demonstrating a deficient mitochondrial respiration chain and ATP generation. In line with the raising ROS concentration and oxidative stress and perhaps as a compensatory mechanism to recover redox state in the myofiber, superoxide dismutase and catalase activities are boosted in mSOD1 skeletal muscle (Leclerc et al., 2001; Mahoney, Kaczor, Bourgeois, Yasuda, & Tarnopolsky, 2006). Accumulation of misfolded mutant proteins observed in skeletal muscle may also aggravate this phenotype since protein folding control in the ER lumen is a highly energy-demanding process, forcing mitochondrial oxidative phosphorylation and ROS generation (Bhattacharya, Wei, Hamilton, & Chaudhuri, 2014; Loeffler, Picchiarelli, Dupuis, & Gonzalez De Aguilar, 2016; Wei, Bhattacharya, Hamilton, Jernigan, & Chaudhuri, 2013).

Besides SOD1, animal models carrying mutations in other ALS-related genes present in sporadic ALS cases, such as vesicle-associated membrane protein-associated protein VAPB/ALS8 also show energy deficits. VAPB mutants have reduced ATP levels and promote

myofiber fat infiltration through FoxO pathway, suggesting the activation of metabolic changes to compensate for mitochondria dysfunction (Han et al., 2013).

In line with the pivotal role of defective mitochondrial respiratory chain and oxidative stress in ALS skeletal muscle, increasing levels of PGC-1 alpha, a transcription coactivator that promotes mitochondrial biogenesis, can improve muscle function even at late stages of the disease (Thau et al., 2012). In this same line, genetic ablation of Parkin, a mitochondrial ubiquitin ligase and negative regulator of PGC-1 alpha, attenuates motor neuron loss and denervation in the SOD1-G93A mouse model of ALS, partially rescuing mitochondrial dysfunction and PGC1 alpha expression, at least in spinal cord (Palomo et al., 2018).

Other than underfeeding, oxidative stress and mitochondrial dysfunction, the origin of the hypermetabolic state and elevated resting energy expenditure observed in ALS still remains obscure, especially considering the loss of mass and function of skeletal muscle, the highest energy-consuming tissue of the body, along disease course (Bouteloup et al., 2009; Ferri & Coccurello, 2017; Kasarskis et al., 2014).

Nevertheless, mapping of denervation patterns in SOD1-G93A mice shows that fast-fatigable motor units innervating myofibers with type II glycolytic metabolism are particularly susceptible for degeneration even before motor symptoms (Frey et al., 2000; Hegedus, Putman, Tyreman, & Gordon, 2008). Skeletal muscle is a major glucose storage in the form of glycogen, that is transformed into ATP through glycolysis. The dysfunction of fast-twitch type IIb myofibers in ALS is consistent with glucose intolerance and insulin resistance reported in ALS patients (P. F. Pradat et al., 2010; Reyes, Perurena, Festoff, Jorgensen, & Moore, 1984) and preferential metabolic shifting toward free-fatty acid beta-oxidation rather than glycolysis, observed in mSOD1 mice models before disease onset (Scaricamazza et al., 2020). Increased levels of pyruvate dehydrogenase kinase 4 (PDK4) and inhibition of pyruvate dehydrogenase complex (PDH) and phosphofructokinase 1 (PFK1) leads to glycogen accumulation and metabolic switch towards lipid catabolism and consequently, an increase of reactive oxygen species that aggravate the mitochondrial dysfunction (Gabriella Dobrowolny et al., 2018; Palamiuc et al., 2015). Reverting this change by daily administration of dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase 4 activity, to SOD1-G86R mice facilitates the entry of pyruvate into the Krebs cycle and glycolysis, restoring PGC-1 alpha and its target gene *Mfn2*, protecting muscle mitochondria and preventing denervation and muscle atrophy (Gabriella Dobrowolny et al., 2018; Palamiuc et al., 2015).

In line with the preferential fatty acid oxidation in skeletal muscle from ALS patients, gastrointestinal absorption of lipids and peripheral clearance of triglyceride-rich lipoproteins were markedly increased in these subjects. Accordingly, high fat diets exert extended survival by 20% in mutant SOD1 mice and ameliorated energy over-expenditure and disease outcomes in ALS patients (Dupuis, Oudart, René, Gonzalez de Aguilar, & Loeffler, 2004; Fergani et al., 2007; Mattson, Cutler, & Camandola, 2007; Oliván et al., 2014).

On the contrary, ablation of the AMP-activated protein kinase (AMPK) in skeletal muscle from wildtype mice promotes gait disturbances that resemble those observed in mSOD1 mice (Vergouts et al., 2015). AMPK acts as an energy sensor, activating both fatty acid oxidation and mitochondria biogenesis but also glycolysis upon muscle ATP depletion (Kjøbsted et al., 2018). Therefore, detrimental effects of AMPK reduction in ALS are likely to

reflect accelerated hypermetabolism and energy deficit through inhibition of both catabolic pathways.

Phosphorylated TDP-43 aggregates are also present in skeletal muscle biopsies from ALS patients (Mori et al., 2019), and this protein is proposed to participate somehow in energy balance homeostasis in this tissue. In this line, myofibers from transgenic mice over expressing wild-type TDP-43 show impaired insulin mediated glucose uptake (Stallings et al., 2013) and postnatal conditional TDP-43 knockout mice exhibit weight loss and fat depletion, confirming that metabolic changes in ALS skeletal muscle are not only associated to mSOD1 pathology.

Metabolism of alternative sources of ATP such as creatine phosphate is also impaired with 67% decrease in mitochondrial creatine kinase activity, at least in spinal cord from mSOD1 mice (Wendt et al., 2002). This deficit cannot be restored by creatine monohydrate intake, although muscle atrophy is partly mitigated (Derave et al., 2003). Unfortunately, creatine monohydrate did not extend survival nor improved fatigue during isometric contraction in ALS patients (Table1) (Rosenfeld et al., 2008).

Taking together these data indicate the existence of an adaptative switch towards oxidative metabolism together with disruption of the main mechanisms for cellular ATP production in ALS skeletal muscle. However, from the therapeutic perspective, mitochondrial co-factors and modulators of oxidative phosphorylation have failed to provide clinical evidences (Table 1), which highlights the need for a better understanding of the underlying origin of mitochondrial impairment, oxidative stress and energy imbalance in ALS. This is especially relevant in skeletal muscle, since this tissue may account up to 60% oxygen and nutrient body consumption, and muscle atrophy represents one of the earliest events in the disease.

PROTEOSTASIS IN ALS MUSCLE

In adults, skeletal muscle mass is dynamically regulated through balance between protein synthesis and degradation mechanisms in combination with signalling networks for protein quality control (PQC), trafficking and clearance. These are all tightly interconnected mechanisms to regulate protein homeostasis, or proteostasis (for review see (Anthony, 2016).

Muscle protein synthesis is regulated at translational level and relies on the formation of two intracellular structures, the ternary initiation complex (composed of methionyl tRNA, eukaryotic initiation factor (eIF) 2 and GTP) and the eIF4 ribosomal complex. Phosphorylation of eIF2 and eIF4 controls the assembly of these complexes and, therefore, the protein synthesis in adult skeletal muscle (Jefferson & Kimball, 2001). On the other hand, proteolytic systems relevant for skeletal muscle mass are mainly the autophagy lysosomal, the ubiquitin proteasome pathway and the unfolded protein response (UPR). Besides skeletal muscle mass, these degradative mechanisms, together with the molecular chaperones such as heat shock proteins (HSPs), also conform the PQC system to target misfolded proteins for degradation or refolding and prevent aggregation and toxicity (Anthony, 2016; Tipton, Hamilton, & Gallagher, 2018). Failure in any of these mechanisms has been linked to denervation, atrophy and muscle wasting (Sandri, 2013).

Skeletal muscle is typically more efficient in activating the proteolytic and PCQ mechanisms compared to nervous system (Onesto et al., 2011). Impairment in several proteostatic mechanisms has been reported in skeletal muscle of patients with neuromuscular conditions such as ALS. Regarding protein anabolic mechanisms, a translational repression

and, therefore, a reduced protein synthesis is associated to many mutant ALS-proteins including SOD1, TDP-43, FUS, and C9ORF72 (Kamelgarn et al., 2018; Kanekura et al., 2016). The exact causes of this blockade remain to be elucidated although stress granule formation and sequestering of mRNAs and translation initiation factors, including eIF2, within mutant protein aggregates have been demonstrated (Cestra, Rossi, Di Salvio, & Cozzolino, 2017).

To the best of these authors' knowledge no studies have been carried out in the search for stress granules in skeletal muscle from ALS patients or experimental models. However, dipeptide repeat (DPRs) inclusions, have been described in skeletal muscle from mutant C9orf72 ALS patients and myocytes differentiated from induced pluripotent stem cells, and are particularly associated to atrophic myofibers (M. D. Cykowski et al., 2019; Lynch et al., 2019). These dipeptide repeats are formed by repeat-associated non-ATG (RAN) translation and in studies carried out in brain sections from ALS patients, one of these repeats (proline-arginine) has been shown to complex with more than a hundred ribosomal proteins and translation initiation factors restraining the access to mRNA and impeding protein translation (Kanekura et al., 2016).

The main catabolic mechanism that targets misfolded proteins is autophagy, which also conducts bulk degradation and recycling of cellular material and organelles. Under normal diet, autophagic markers such as Beclin1 (BECN1/ATG6), microtubule-associated protein LC3 (MAP1LC3A) and sequestosome 1 (SQSTM1/p62) and autophagosome formation have been found upregulated in muscle from mSOD1 mice (Crippa et al., 2013; G. Dobrowolny et al., 2008; Oliván et al., 2015; Y. Xiao et al., 2015). Starvation stress boosts autophagic flux, increasing autophagosome and lysosome formation and, thus, vesicle cargo degradation in skeletal muscle of wild type mice but not in that of mSOD1 mice (Y. Xiao et al., 2015). This could be explained by a caspase 3 -dependent cleavage of BECN1 autophagy promoter observed in this ALS model (Y. Xiao et al., 2015). Similarly, increased p62 and LC3 protein levels and autophagosomes in cell cultures under nutrient rich medium but failed activation of autophagosome formation after stress-induced starvation in fibroblasts from ALS patients carrying the C9orf72 repeat-expansion (Aoki et al., 2017).

Mutations in a gene encoding the valosin-containing protein (VCP) can also cause familial ALS. VCP is a member of the ATPase family critical for protein homeostasis in muscles and neurons (Johnson, Shu, Hauswirth, Tong, & Davis, 2015). Chemical or genetic inhibition of VCP interfere with the maturation of autophagosomes leading to accumulation and hampering the fusion with lysosomes. In VCP-mutant mouse skeletal muscle there is a diminished activity of mTOR, an inhibitor of autophagosome formation, and this is concomitant with upregulation of autophagosome biogenesis. A further inhibition of mTORC1 activity with rapamycin exacerbated weakness, muscle atrophy and vacuolation and increased p62 and LC3-positive vesicle accumulation in skeletal muscle from VCP-mutant mice (Ching et al., 2013), suggesting that mutations in VCP impair autophagosome degradation but no biogenesis. In line with these findings, the loss of a novel VCP interactor Washc4/Swip (WASH complex subunit 4) in the vertebrate model zebrafish alters autophagy-mediated proteostasis in striated muscle cells (Kustermann et al., 2018).

Taken together these data show compelling evidence that gene mutations found in ALS patients impair different stages of the autophagy flux, and this phenotype is exacerbated upon stress induced by starvation or rapamycin treatment. In scenario, therapeutic interventions in ALS models to simply stimulate autophagic flux might be detrimental, enhancing undigested autophagosome cytoplasmic accumulation.

Indeed, in the mutant FUS fly model of familiar ALS, overexpression of an ankyrin-repeat and KH-domain containing protein Mask, enhanced autophagosome degradation through lysosomal acidification, ameliorating protein aggregation in muscle from these flies (Zhu, Zhang, Tian, & Wu, 2017). Human ortholog of Mask ANKHD1, is highly expressed in skeletal muscle and its function in involved in endosomal maturation (Kitamata, Hanawa-Suetsugu, Maruyama, & Suetsugu, 2019).

The unfolded protein response (UPR) is a protein quality control mechanism that promotes refolding or degradation of misfolded proteins to restore the physiological equilibrium within the cell. Accumulation of aberrant proteins in endoplasmic reticulum (ER) lumen leads to stress and triggers the UPR by inducing expression of heat shock protein chaperones (HSPs) to refold the proteins or proteasome degradative pathway via polyubiquitin chain tagging of the target protein. Failure or overwhelming of these pathways leads to UPR-induced cell apoptosis (Kaganovich, Kopito, & Frydman, 2008).

ER stress and UPR modulation by mutant ALS proteins have been reported in skeletal muscle from SOD1 ALS models (Chen, Wang, & Chin, 2015; Loeffler et al., 2016). In this sense, we observed a significant upregulation of proteasome activity was at pre-symptomatic stage, while symptomatic and terminal stages showed a reduction in this pathway in skeletal muscle from mSOD1 mice (Oliván et al., 2015). Likewise, ubiquitin-positive inclusions have been also detected in muscles from C9ORF72 ALS patients, suggesting an implication of the ubiquitin proteasome pathway in the pathogenesis of the C9-related ALS (Maurel et al., 2018). Small heat shock proteins such as B8 (HSPB8), are also highly expressed in skeletal muscle from ALS models and might contribute to autophagy activation and protein aggregate clearance (Rusmini et al., 2017) in agreement with a mutual interplay between autophagy and the UPR. This hypothesis is supported by overexpression of viral and Drosophila homologs of HSPB8, ICP10PK and HSP67Bc respectively, to preserve neuromuscular junction and ameliorate disease outcomes in the SOD1-G93A mice (Aurelian, Laing, & Lee, 2012; Ritson et al., 2010).

Further evidences of UPR induction and autophagy regulation upon mutant ALS-protein expression have emerged from *in vitro* experiments where the C2C12 mouse myoblasts and mouse embryonic fibroblasts were transfected with mutant versions of VAPB and ER-Sigma receptor-1 (SIGMAR1) respectively, showing UPR activation, protein aggregation and autophagy impairment (Dreser et al., 2017; Y. Tokutake et al., 2015).

Epigenetic regulators have also been quantified within skeletal muscle from ALS patients. Transcript levels of histone deacetylase 4 (HDAC4) are upregulated in patients with fast disease progression and HDCA4 protein levels negatively correlating with the extent of reinnervation observed on muscle biopsies from patients (Bruneteau et al., 2013). By altering acetylation of histones, HDACs can modulate gene expression of UPR proteins, regulating the levels of heat shock chaperones, decreasing ubiquitination and proteasomal degradation. Interestingly, skeletal muscle restricted knockout of HDAC4 in mSOD1 mice results in levels of poly-ubiquitinated proteins and proteasome activity similar to those of wildtype control animals (Pigna et al., 2019). A summary of abnormalities in proteostasis mechanisms observed in ALS is depicted in Figure 2.

From the therapeutic perspective, promising strategies are based on increasing the content of neurotrophic factors in ALS muscle to counteract the impairment in proteostasis and to maintain muscle physiology. This is the case of insulin-like growth factor 1 (Igf1), which stimulates growth and development through satellite cell myogenesis but also increasing protein synthesis mediated by Akt-mTOR and inhibiting autophagy (see (Vainshtein & Sandri, 2020)for review). The upregulation of Igf1 in muscle, using recombinant plasmid injections or MLC/mIgf1 mouse (which selectively expresses mouse Igf1 under the transcriptional controls of myosin light chain, MLC, promoter in fast-twitch fibers) cross breeding with mSOD1 mice can improve hindlimb muscle strength, as well as increase motor unit and motor neuron survival (Gabriella Dobrowolny et al., 2005; Riddoch-Contreras et al., 2009). The use of subcutaneous implants of dihydrotestosterone in mutant SOD1-G93A mice at early symptomatic age also induces increasing levels of Igf1, attenuates neuromuscular junction denervation and improves motor behaviour, increasing the lifespan in these animals (Yoo & Ko, 2012).

In a clinical trial carried out in North America, subcutaneous recombinant human IGF1 delayed functional impairment and symptom progression as measured by AALS total score (Lai et al., 1997). However, a phase 3 and a 2-year placebo-controlled trial in European patients did not corroborate these results, failing to detect changes in muscle strength or function (Borasio et al., 1998; Sorenson et al., 2008). Unfortunately, lack of information on the IGF1 concentration in skeletal muscle during these clinical trials and differences in doses, timeframes and systemic rather than muscle-targeted delivery systems for IGF1 in pre-clinical versus clinical trials hamper comparison of results among these studies (Table 1). Conversely, other therapeutic approaches have been designed to assist refolding or clearance of ALS-linked proteins i.e. by autophagy or UPS initiation or enhancement of heat shock protein or chaperone translation in patients (Table 1). The use of recombinant plasmids to overexpress a selective chaperone (HSPB8) and co-chaperones (BAG1 and BAG3) can modulate autophagy and proteasome by removing TDP-25 (the C-terminal 25 kDa fragment of TDP-43) inclusions in immortalized motor neuron -like (NSC34) cells and stabilized myoblasts (Cicardi et al., 2018).

These data highlight the therapeutic potential of regulating proteostasis network in ALS skeletal muscle. However, they also remark the need to finely monitor drug concentration in skeletal muscle and proteins or nucleic acids targeted since these mechanisms are already stressed and challenged by misfolded proteins, abnormal organelles and aggregated nucleic acids and peptides. Excessive boosting of autophagy and UPR or without further facilitation in the clearance of the cargo might cause overload and be detrimental for the overall balance between protein synthesis and degradation and thus contribute to skeletal muscle atrophy and denervation in ALS.

RNA METABOLISM IN ALS SKELETAL MUSCLE

Products of approximately half of the known ALS genes are involved in regulation of RNA processing, including transcription, splicing, stabilization, RNA trafficking and processing of non-coding RNAs (Butti & Patten, 2019). Among these, C9orf72, SOD1, TDP-43 and FUS mutations count for 40-50% of the genetic ALS cases. Many of the ALS-linked proteins, such as TDP-43, FUS, HNRNPs A1 and A2B, TAF15, EWSR1, MATR3 and TIA1, are RNA binding

proteins (RBPs) containing one or more RNA recognition motifs involved in their normal biological function. These proteins generally also contain low complexity, or prion-like domain that allows phase separation to microdomains where RNA processing takes place. Indeed, many of ALS-linked proteins are found to accumulate in RNA foci such as cytoplasmic stress granules or nuclear paraspeckles, both structures being membrane-less organelles implicated in ALS (An, Tan, & Shelkovnikova, 2019). This general property provides a possible reason for broad scale impact on RNA metabolism by the ALS-causing mutations. However, not all ALS genes are RBPs by nature.

Some ALS-linked mutations, such as those in SOD1, may induce pathological RNA binding properties and sequester RBPs or other gene products from their normal biological function (Lu et al., 2007). Similar toxic gain-of-function may result in C9ORF72 cases through hexanucleotide GGGGCC (G4C2) repeat expansion-created RNA species or through dipeptide repeat proteins (DPR) that bind and sequester essential RBPs (Barker, Niblock, Lee, Shaw, & Gallo, 2017). Although our understanding of RNA metabolism in ALS has improved in recent years, relatively little is known on how these processes could be mechanistically involved in skeletal muscle pathology observed in ALS, or indeed, in the etiopathogenesis of the disease itself.

TDP-43 is a nucleic acid–binding protein whose mislocalization and aggregation in neurons represents a widely recognized hallmark in pathology of sporadic and most familial ALS cases. In normal cells TDP-43, a product of TARDBP gene, is mostly localized in the nucleus, notably in paraspeckles, where it primarily regulates transcription and pre-mRNA splicing. However, upon cellular stress and in ALS it localizes to cytoplasmic stress granules or similar inclusions, which may cause dysregulation of its normal nuclear targets (Zhao, Kim, van Bruggen, & Park, 2018). TDP-43 is essential for *in vitro* skeletal muscle cell differentiation and required for skeletal muscle regeneration *in vivo* (Vogler et al., 2018). Interestingly, phosphorylated TDP-43 has been shown to aggregate in the skeletal muscle of sporadic and familial ALS samples (Matthew D. Cykowski et al., 2018), although is not yet clear whether the aggregation and mislocalization plays an active role in the disease or reflects the altered homeostatic response to the neuronal pathology. Supporting the active role in normal muscle function, the Drosophila ortholog of TDP-43 promotes the formation and growth of neuromuscular synapses and its muscle-specific downregulation greatly decreases the locomotor capacity and lifespan (Strah et al., 2020). Analogous to the described role of TDP-43 in local translation of mRNA in neurons (Alami et al., 2014), it was recently demonstrated that TDP-43 forms cytoplasmic, mRNA containing amyloid-like assemblies during normal regeneration of skeletal muscle (Vogler et al., 2018). These temporary structures that the authors coined myo-granules, contain mRNAs encoding sarcomeric proteins and are lost in fully mature myotubes. Although myo-granules seem to form an essential regenerative response in normal muscle regeneration, it remains possible that conditions associated with TDP-43 mislocalization, such as ALS, may alter the balance of assembly and clearance of these structures in the skeletal muscle.

Similar to TDP-43, FUS protein plays an essential role in mRNA splicing and localization, stress granule formation, and other aspects of RNA metabolism. However, unlike loss of TDP-43, postnatal elimination of FUS has no effect on motor neuron function (Sharma et al., 2016) suggesting that FUS-dependent motor degeneration is a gain of function conferred by ALS mutations. ALS-mutant FUS is intrinsically toxic to both muscle cells and motor neurons,

and in the muscle the toxicity is at least partially attributable to defective transcriptional regulation of acetylcholine receptor subunits in subsynaptic myonuclei (Picchiarelli et al., 2019). As a result, knock-in mice expressing mutant FUS suffer from denervation of neuromuscular endplates before motor neuron loss, which is consistent with a 'dying-back' model. Of note, FUS is a member the structurally similar to FET protein family consisting of three ALS genes (FUS, EWS and TAF15) all of which are present in skeletal muscle and mutations in which can cause muscle defects involving mitochondrial function (Picchiarelli & Dupuis, 2020). FUS is also an essential component of nuclear paraspeckles and can be found in recently discovered nuclear stress-induced silencing complex responsible for nuclear transport of small nucleotide sequences, including miRNAs (Castanotto et al., 2018). Finally, FUS is involved in optimal miRNA-mediated gene silencing as a component of cytosolic miRISC, where it interacts with the core miRNA-binding proteins of Argonaute (AGO) family. However, whether to what extent these specific foci are affected by FUS mutations in ALS muscles is currently unknown.

Hexanucleotide repeat expansion in C9ORF72 gene results in production of G4C2 repeat RNA and DPR derived from these transcripts, and both of these products may sequester low-complexity domain-containing proteins that have critical function in RNA metabolism and in the formation of cytoplasmic and nuclear RNA foci. G4C2 repeat-associated RNA foci and DPR are present in the skeletal muscle of at least in fly (Freibaum et al., 2015) and zebrafish (Shaw et al., 2018) models of C9ALS. Although the main target of the fly studies was the central nervous system, it was shown that muscle-specific expression of G4C2 causes age-dependent flight muscle defects as witnessed permanent abnormal wing position (Freibaum et al., 2015). Recent studies have revealed the presence of DPRs in C9ALS patient muscle (M. D. Cykowski et al., 2019) and increased amount of RNA foci in iPSC-derived skeletal myofibers from C9ALS patients (Swartz et al., 2016). In the latter case, the C9orf72 repeat expansion did not affect the efficiency of myoblast differentiation compared with controls cells, suggesting a limited role for skeletal muscle physiology *in vitro*. However, more studies are warranted to investigate if the expression of mutant C9ORF72 transcripts specifically in skeletal muscle *in vivo* could contribute to the ALS phenotype.

Like the native product of C9ORF72, SOD1 does not contain RNA-binding motifs. However, as a result from a gain-of-function due to abnormal exposure of parts of the SOD1 polypeptide that can bind nucleic acids, misfolded mutant SOD1 can bind specific mRNAs to regulate their expression or stability by selectively binding AU-rich elements (AREs) found in the 3' untranslated regions (Lu et al., 2007). The target mRNAs of misfolded SOD1 include vascular endothelial growth factor VEGF whose overexpression in skeletal muscle has been shown to be beneficial in mutant SOD1 mice (Azzouz et al., 2004). Remarkably, the interaction of mutant SOD1 with VEGF mRNA also results in abnormal recruitment of other ARE-binding proteins (Li et al., 2009) such as ELAVL1/HuR that normally facilitates muscle regeneration by promoting expression of myogenic factors MyoD and myogenin. Thus, mutant SOD1-initiated titration of protective factors such as ELAVL1/HuR may impair post-transcriptional regulation by sequestering key regulatory RNA-binding proteins from their normal targets. In turn, this could contribute to the previously described truncated myogenic response in SOD1-G93A mice, where an induction is seen in RNA but not in protein level (Manzano, Toivonen, Olivan, et al., 2011).

MicroRNAs regulate gene expression post-transcriptionally through the binding to their specific target mRNAs and promoting mRNA degradation or translational repression. This regulation relies on a cytoplasmic miRNA-induced silencing complex (miRISC), a ribonucleoprotein complex that incorporates the miRNA and uses it as a template to recognize the target transcripts. Several miRNAs have been described altered in skeletal muscle in ALS and its models (Di Pietro, Lattanzi, & Bernardini, 2018). Most notably, denervation-inducible, skeletal muscle –enriched miR-206 has been shown to progressively increase with the advancing pathology in the fast twitch (but not slow twitch) muscles of SOD1-G93A mice (Toivonen et al., 2014). The presence of miR-206 has been shown to protect ALS mice by promoting NMJ stabilization, at least partially through inhibition of histone deacetylase HDAC4 and the following induction of FGF signalling to stimulate reinnervation (Williams et al., 2009). In agreement, muscle biopsies from patients with genetic forms of ALS show strong upregulation of miR-206 and a decreased expression of HDAC4 protein levels (Pegoraro, Marozzo, & Angelini, 2020). However, like in the case or MRF where the transcript levels do not translate in the increased protein levels (Manzano, Toivonen, Oliván, et al., 2011), the upregulation of miR-206 is not sufficient to inhibit ultimate denervation accompanied with ALS. One of the potential reasons could be that its incorporation into functional miRISC complexes might be affected by the disease. Interestingly, TDP-43 and FUS interact with the core miRNA-binding proteins of Argonaute (AGO) and are directly involved in the miRISC formation. Furthermore, TDP-43 has been shown to specifically disrupt miR1/miR-206 incorporation with miRISC in muscle cells, thus dampening its biological activity (King et al., 2014). Although the role of TDP-43 mutations associated with ALS have not been yet evaluated in the context of such miRNA damping, it remains plausible that documented cytoplasmic accumulation of TDP-43 in the context of most ALS mutations may have direct consequences on reinnervation capacity of ALS muscles through modulation of retrograde signalling (HDAC4-FGF axis) to the NMJ.

Finally, since many characterized ALS-causing genes encode RBPs it is not surprising that also other RNA species that interact with these proteins might participate in ALS onset or disease progression. Long-noncoding RNAs (lncRNAs) regulate gene expression through diverse mechanisms, including modulation of transcription, direct antisense-mediated mRNA stabilization or destabilization, inhibition or stimulation of protein translation through modifications of ribosome activity, and indirect stabilization of mRNAs through miRNA sequestration (sponging). Additionally, many lncRNAs function as scaffolds to bring together multiple proteins to form ubiquitous RNP complexes such as paraspeckles and stress granules. One of the lncRNAs deregulated in ALS is NEAT1, a paraspeckle scaffold RNA that has been shown to bind ALS-linked proteins such as TDP-43, FUS and MATR3 (Banerjee, Vest, Pavlath, & Corbett, 2017; Nishimoto et al., 2013). NEAT1 levels are induced during myoblast differentiation, which suggests that NEAT1 and augmented paraspeckle activity may be critical for normal muscle cell function and regeneration. In skeletal muscle *in vivo*, MATR3 is essential for myogenesis and directly regulates the levels NEAT1. Regarding to other non-coding RNAs, circular RNAs (circRNAs) can also serve as RNA-miRNA sponges or as scaffolds for RNA-binding proteins. At this point, only one study has explored the possibility of differential circRNA regulation in ALS, and several candidates were found in blood of ALS patient-derived samples (Dolinar, Koritnik, Glavač, & Ravnik-Glavač, 2019). circRNAs are generated by back-splicing during pre-mRNA processing, and are resistant to RNA exonucleases. The circular form confers them highly stable in cells, which is a desired

feature for biomarker purposes. However, to date, there is no information on potential circRNA modifications in solid tissues such as nervous system or skeletal muscle.

DEFECTS IN MUSCLE REGENERATION AND RESIDENT STEM CELLS

In adults, myogenesis occurs after a stimulus that challenges skeletal muscle structure or function, that is denervation, intense exercise, injury of disease. It relies on the activation and differentiation of tissue-committed stem cells, so-called satellite cells. Satellite cells are mitotically quiescent and positive for Pax7, but after receiving the appropriate stimuli, enter cell cycle to become myoblasts and undergo multiple rounds of division. Part of this progeny will return to quiescence as a self-renewal mechanism to maintain the satellite cell pool, while others will proceed to terminal differentiation and fusion with each other to form multinucleated myofibers or with the parental myofiber to repair the injury. These events are controlled by a series of transcription factors called myogenic regulatory factors (MRFs). The first identified was myogenic determination factor 1 (MYOD1) and latter myogenic factor 5 (MYF5), myogenin, and herculin (MRF4). MRFs form homodimers and heterodimers and through their basic helix-loop-helix domain (bHLH) bind specific sequences in DNA called E-boxes (CANNTG) that are ubiquitously found in promoter and enhancer regions of different muscle and non-muscle specific genes. Even if other muscle-resident stem cells like mesenchymal or adipose-tissue-derived cells have been shown to possess the ability to enter the myogenic program upon the appropriate stimuli, their actual contribution to in vivo muscle repair and regeneration remains controversial (Madaro et al., 2018).

In ALS, evidences from different groups have demonstrated an impairment in skeletal muscle satellite cell activity and myogenic potential. In this sense, when the P56S mutation in VAPB gene, associated to ALS type-8 (ALS8)(Nishimura et al., 2004), is introduced in the murine C2C12 myoblast cell line, the IRE1-XBP1 pathway (which is involved in the unfolded protein response) is impaired and aberrant myonuclear location is observed, which is associated with a reduced myotube formation in these cells (Yukako Tokutake et al., 2015).

In this line, in the ALS mouse model SOD1-G93A our group has previously described a progressive upregulation in Pax7 and myogenic regulatory factors transcripts (MyoD1 and Myogenin) in skeletal muscle (Manzano, Toivonen, Olivan, et al., 2011). Moreover, in satellite cells harvested from these animals, we were first to report a reduced proliferative capacity from presymptomatic disease stages (Manzano et al., 2013). This was accompanied by a reduction in the number of Pax7-positive satellite cells and increased number of activated (MYOD1-positive) cells per myofiber (Manzano et al., 2012). Interestingly, a different pattern was observed in myofibers isolated from fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus (SOL) muscles, which is in agreement with the longer resistance of this last myofiber type to denervation and weakness induced muscle atrophy in ALS.

In ALS patient samples, using quantitative electron microscopy in skeletal muscle biopsies, Ishimoto et al. reported an increased euchromatin percentage in satellite cells (indicative of an active transcription and cell cycling) but without an increment in satellite cell numbers (Ishimoto, Goto, Ohta, & Kuroiwa, 1983). This is in agreement with a reduction in transcripts

for *PAX7* and *MYF5* myogenic markers observed by Jensen et al. and with the majority of those PAX7 positive satellite cells being negative for MYOD (Jensen, Jørgensen, Bech, Frandsen, & Schrøder, 2016). Other, immunohistochemical analyses have also revealed various degrees of denervation- and reinnervation with satellite cell activation (P.-F. Pradat et al., 2011). These data are thus indicative of in vivo activation of satellite cells in ALS patient skeletal muscle but without a progression through the myogenic program.

This was confirmed in vitro when only a subset of these cells were able to generate colonies when harvested and put in culture, displaying an altered senescent-like morphology and increase of senescence associated βGal activity and CDKN2A/p16 expression, with a large number of vacuoles (P.-F. Pradat et al., 2011). Besides, for those cultures viable, ALS-derived satellite cells are unable to fully differentiate as shown by abnormal myotube morphology and reduced MYF-4 and myosin heavy chain isoform 1 gene and protein expression compared to controls (P.-F. Pradat et al., 2011; Scaramozza et al., 2014).

Besides MRFs, myogenesis is regulated post-transcriptionally by muscle-specific microRNAs, so-called myomiRs. MicroRNAs are short (18-22 nt) oligonucleotides that bind the 3'untranslated region of specific target mRNAs, inducing their degradation or blocking translation. A subset of them (miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, and miR-499) are striated muscle-specific, with miR-206 and miR-133b expression restricted to skeletal muscle, and miR-208a to cardiac muscle. This specificity is due to their genomic location within introns of myosin heavy chain genes or their transcriptional control by muscle specific transcriptional factors such as MyoD1. Importantly, myomiR functions partially overlap and regulate myoblast proliferation and differentiation, muscle regeneration, or fibre-type specification.

In EDL fast-twitch muscles (preferably affected in the disease) from SOD1-G93A mice, our group has previously reported an increase in mature miR-206, with highest expression towards the most severely affected animals (Toivonen et al., 2014). Moreover, microRNA-206 depletion was latter shown to accelerate disease progression and diminish survival by ~1 month in the SOD1-G93A mouse, and this was accompanied by exacerbated muscle atrophy (Williams et al., 2009). These authors also demonstrated that miR-206 upregulation protects neuromuscular junctions after nerve injury promoting reinnervation, which may contribute to its beneficial effects in ALS. These results were confirmed skeletal muscle biopsies from patients with genetic forms of ALS (carrying mutations in C9orf72 and SOD1 genes), where elevated levels of miRNA-206 were found, together with miR-23a, miR-29b, miR-31, and miR-455 (Russell et al., 2013).

In line with the contribution of incomplete muscle regeneration and stem cell pathology to disease progression in ALS, several therapeutic strategies have targeted this mechanism.

In the SOD1-G93A mouse, muscle-restricted expression of insulin-like growth factor-1 isoform preserved myofiber structure and enhanced satellite cell regeneration, activating calcineurin-mediated pathways and anti-apoptotic Bcl-2 and reducing Caspase-1. This was accompanied by reduced motor neuron death, delayed disease onset and progression, extending the live span of the animals by 30 days (G. Dobrowolny et al., 2008; Gabriella Dobrowolny et al., 2005). However, it cannot be ruled out that diffusion of IGF1 secreted by

skeletal muscle into nearby NMJ and its known retrograde signalling into motor neurons (Caroni & Grandes, 1990) contribute to these findings. Indeed, reduction of mutant SOD1 expression exclusively in skeletal muscle, by gene excision, lentivirus- or AAV-encoded siRNA to SOD1 did not alter disease onset, progression or survival in these mouse models (Miller et al., 2006).

In a different study, activation of P2X7 ionotrophic receptor in the SOD1-G93A mouse skeletal muscle stimulated the myogenic program from satellite cells and preserved the morphology of neuromuscular junctions, reducing denervation and atrophy of skeletal muscles in these mice (Fabbrizio et al., 2019). Granulocyte colony-stimulating factor (GCSF/CSF3) is a cytokine used to prevent neutropenia in patients receiving myelosuppressive anticancer drugs and has been also shown to increase neurogenesis and neuroplasticity and boost muscle regeneration in Duchenne muscular dystrophy mice. In our experience, SOD1-G93A mice treated with GCSF subcutaneously showed delayed disease onset, improved motor performance and prolonged survival compared to non-treated controls and this was followed by reduced neuromuscular junction dismantling and an increase in satellite cell proliferation (Rando et al., 2017). Following a different approach, cell therapy implants of mesenchymal stem cells (MSCs) in skeletal muscle of ALS mice and rats increased longevity with improved motor function and decreased motorneuron degeneration in the spinal cord. These effects have been explained by secretion of glial-derived neurotrophic factor and neurotrophin 4 by MSCs in the grafted muscles and the neuromuscular junction (Rando et al., 2018; Suzuki et al., 2008). Taking together these results suggest that stimulation of satellite cell activity and muscle regeneration may be therapeutic in ALS patients. Mesenchymal stem cells have been also transplanted into skeletal muscles of ALS patients demonstrating engraftment and low toxicity (Table 1). Beyond neurotrophic supply, transplanted cells have been suggested to regulate inflammation in skeletal muscle.

In this sense, a finely tuned cross-talk between immune system and muscle-resident stem cells is necessary for muscle regeneration. Besides satellite cell activation and proliferation, muscle injury promotes recruitment of neutrophils, monocytes and CD8+ T cells to the site of trauma through the release of cytokines and CC-chemokine ligand 2 (CCL2) and CXC-chemokine ligand 1 (CXCL1) originating a primary inflammatory response orchestrated by interferon-γ (IFNγ) and tumour necrosis factor (TNF). Cytokines also induce the expression of MRFs genes by the myogenic progenitors, that together with IGF1 released by M1-macrophages contribute towards the satellite cell proliferation to expand the myogenic pool (for review see (Tidball, 2017)). A further production of amphiregulin by regulatory T cells increases the expression of myogenic-regulatory factors that drive the later stages of the myofiber differentiation and maturation (Schiaffino, Pereira, Ciciliot, & Rovere-Querini, 2017). Other cells present in skeletal muscle interstitium such as fibroadipogenic progenitors (FAPs) and regulated by transforming growth factor-β (TGFβ) (from macrophages) and by IL-4 (from eosinophils), differentiate into fibroblast to produce extracellular matrix components that will be the scaffold for the new myofiber and release IGF1 to promote its growth (Heredia et al., 2013; Ismaeel et al., 2019).

In ALS, macrophage infiltration and increased inflammatory cytokines (IL-1β and TNF-α) have been reported in skeletal muscle from patients and rat models, (Van Dyke et al., 2016). Moreover, terminal complement activation has been observed in tibialis anterior from

SOD1-G93A mice, with increased levels of C1qB, C4, fB, C3 and C5a, contributing to recruitment of C5aR1-positive macrophages into this tissue (H. A. Wang, Lee, Lee, Woodruff, & Noakes, 2017). Interestingly, genetic ablation of C5ar1 gene or systemic inhibition of C5a–C5aR1 signalling ameliorated disease pathology, reduced hind limb weakness and paralysis and extended the survival of these mice (Trent M. Woodruff et al., 2008; T. M. Woodruff, Lee, & Noakes, 2014).

Accumulation of degranulating mast cells associated with neutrophils have been also observed in myofibers and motor endplates from ALS patients and SOD1-G93A rats, and have been suggested to contribute to denervation (E. Trias et al., 2017; Emiliano Trias et al., 2018). Consistently, treatment of symptomatic SOD1-G93A rats with Masitinib, a tyrosine kinase inhibitor, prevented mast cell and neutrophil infiltration in skeletal muscle, axonal pathology, secondary demyelination, and the loss of type 2B myofibers (Emiliano Trias et al., 2018). Of note, a double-blinded and placebo-controlled Phase 2/3 clinical trial recently confirmed a reduced functional decline as measured by ALSFRS-R scale, in non-fast progressing ALS patients (Table 1) (Mora et al., 2020).

On the other hand, upregulation of TGFβ levels and increase in the numbers of FAPs in skeletal muscle have been observed in SOD1-G93A mice correlating with myogenesis deficiencies and fibrosis in this tissue (Gonzalez et al., 2017).

Taking together, these results suggest that inflammation in skeletal muscle of ALS patients and animal models contribute to denervation and dysfunctional muscle regeneration observed in the disease and modulation of inflammatory cytokines and cells represent a promising therapeutic strategy. Since all the previous studies have been carried out using systemic delivery of drugs or ubiquitously expressed gene modifications, it remains to be analysed if and how restricted immune system modulation in skeletal muscle will influence disease pathogenesis.

Physiologically, micro-injuries generated after acute and endurance training have been also shown to activate satellite cells and myogenesis in skeletal muscle, being this response age, gender, fiber-type and exercise dependent. However, the effect of exercise for ALS course has been long discussed (see (Braga, Pinto, Pinto, & de Carvalho, 2018; Kato, Hashida, & Konaka, 2018; Tsitkanou, Della Gatta, Foletta, & Russell, 2019) for review). Some studies have linked physical activity to increased production of reactive oxygen species and oxidative stress, thus worsening the physical condition and motor neuron degeneration in ALS. Others have reported beneficial outcomes with mild to moderate training in ALS patients and animal models (Table 1). In this sense, swimming-based training preserved the number, cross-section area and fiber-type distribution of myofibers in the ALS mice and limited hypotrophy in both slow- and fast- twitch muscles (Deforges et al., 2009).

CONCLUDING REMARKS

Evidences of participation of skeletal muscle in ALS etiopathogeny arise from several perspectives. Pathogenic findings in the disease suggest an early dismantling of NMJ (dying-back) with destruction of postsynaptic apparatus and late axonal and motor neuron cell body degeneration (Fischer et al., 2004; Pun et al., 2006). Moreover, ALS deregulates molecular mechanisms that are critical for skeletal muscle homeostasis including energy handling through mitochondrial respiratory chain (skeletal muscle is a major ATP consumer); proteostasis, which conditions the ratio between protein synthesis and degradation and, thus, maintenance of skeletal muscle mass; and the activity of stem cells responsible for

regenerating a tissue that is constantly stressed by cytoskeletal actin-myosin contraction. Therapeutic approaches targeting the above mentioned pathways in skeletal muscle, have frequently ameliorated disease progression and extended survival in mouse models. Conversely, mutation of genes associated with ALS such as SOD1, restricted into skeletal muscle mimicked disease hallmarks including NMJ disruption, muscle atrophy and MTN degeneration.

The intimate connection between myofibers and nervous system through motor endplate, halts discerning between pathogenic events in each tissue. Moreover, reciprocal interaction between molecular mechanisms in skeletal muscle and systemic conditions such as hormonal, inflammatory and nutritional status or physical activity of the individual impedes to delineate a cause-effect pattern. Nevertheless, gaining insight into molecular mechanisms underlying ALS pathology in skeletal muscle including NMJ rearrangement, muscle atrophy and degeneration will ease novel therapies to ameliorate the disease.

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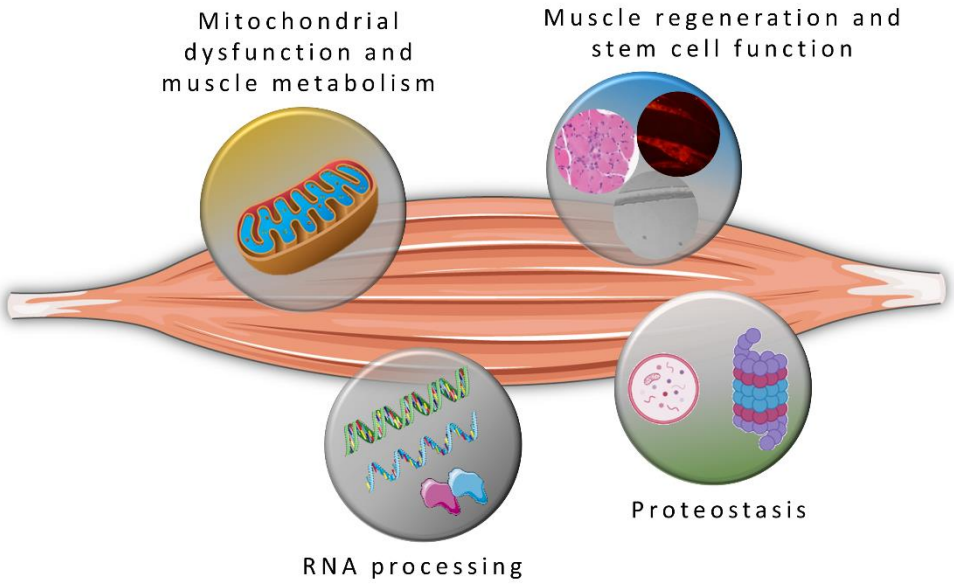

Figure 1. Regulatory network in skeletal muscle that contributes to ALS disease pathology. The image recapitulates the molecular mechanisms involved in skeletal muscle toxicity in ALS as discussed in the text, mitochondrial dysfunction and muscle metabolism, proteostasis, muscle regeneration and stem cell function and RNA processing.

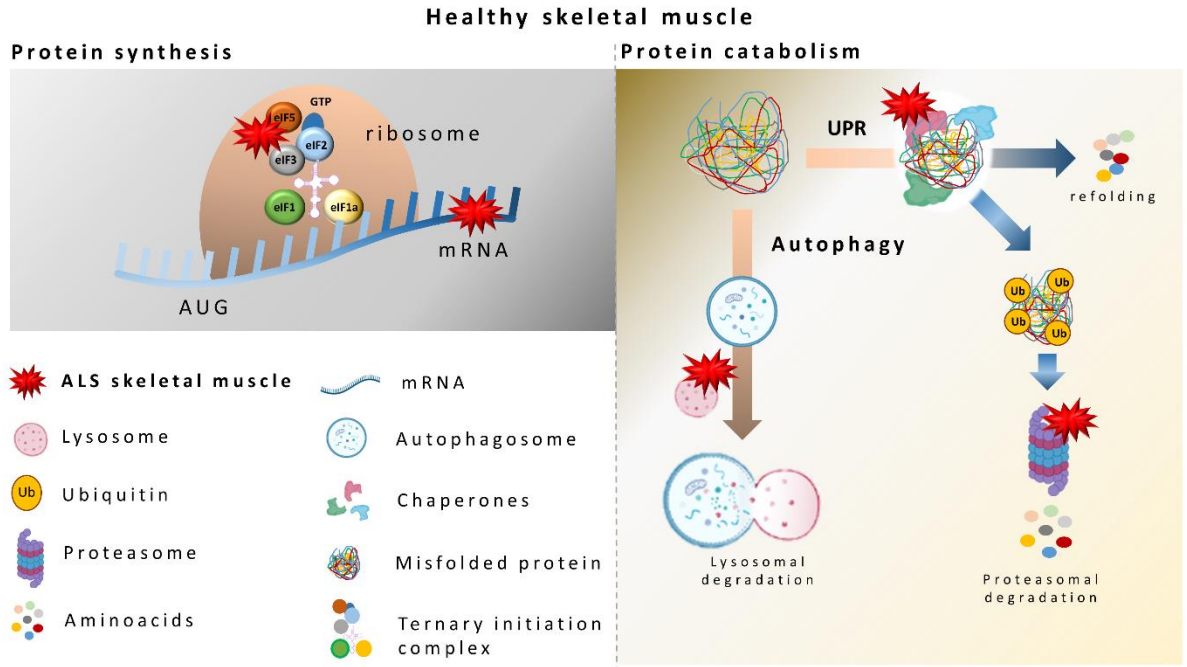

Figure 2. Mechanisms proposed for proteostasis impairment in ALS skeletal muscle. A general translational repression has been demonstrated in ALS, based on sequestering of mRNA and transcriptional machinery components by stress granules and mutant protein aggregates. The reduction in chaperon protein synthesis also halts refolding of mutant misfolded proteins during the unfolded protein response (UPR). Autophagy and proteasomal catalytic pathways show incomplete activation or function based on reduced synthesis of promoters (i.e. Beclin 1) or abnormal autophagolysosomal fusion and cargo degradation. Impairment of UPR and catalytic pathways for misfolded mutant proteins lead to their accumulation and aggregation in a positive feedback manner.

Table 1. **Clinical trials using drugs that target molecular mechanisms altered in ALS skeletal muscle.** According to the National Institutes of Health (NIH) clinical trial database((NIH)), 681 clinical trials have been or are currently under conduction in ALS patients, 366 of them with expected outcomes in skeletal muscle structure or function. This table represents an overview of the clinical trials in ALS using drugs that are known regulators of proteostasis, muscle regeneration and stem cell therapy, mitochondrial function and RNA metabolism.

	Drug/approach	*Phase*	*Mechanism of action*	*Identifier*
Proteostasis	Colchicine	II	Enhancement of translation of Heat shock and other autophagy proteins	NCT03693781
	Rapamycine	II	Autophagy activator	NCT03359538
	Guanabenz	II	inhibition of eIF2α dephosphorylation and stimulation of UPR	(Bella et al., 2017)
	L-Serine	II	ER chaperone translation	NCT03580616 NCT01835782
Muscle regeneration and Stem cell transplantation	Exercise	I and I/II	Resistance, endurance, stretching, Electromyographic stimulation	NCT01521728 NCT03326622 NCT00160004 NCT01650818 NCT03201991 NCT00204464 NCT03076632 NCT01369901 NCT02457715 NCT00956488 NCT01166022
	Endocannabinoid palmitoylethanolamide (PEA)	Not Applicable	Muscle contraction and electrophysiology	NCT02645461
	Masitinib	2/3	Inflammatory modulation	NCT03127267
	Insulin like growth factor, type 1	II and III	Rgeneration/activation protein anabolic pathways	NCT00035815 (Borasio et al., 1998; Lai et al., 1997)
	Mexiletine Dronabinol	Not applicable, II	Muscle cramps and muscle twitching	NCT02781454 NCT00812851
	Bone marrow derived-Mesenchymal Stem Cells	Pilot, I, II and III	Stem cell transplantation	NCT03280056, NCT02017912 NCT02881489 NCT01254539 NCT00855400 NCT02286011

				NCT01363401 NCT03067857 NCT01758510 NCT01777646 NCT01051882
	Umbilical Cord Mesenchymal Stem Cells	II	Stem cell transplantation	NCT01494480 NCT02236065
	Adipose tissue derived-Mesenchymal stem cells	I and II	Stem cell transplantation	NCT03268603 NCT02383654 NCT02492516 NCT03296501 NCT02290886
	Peripheral blood stem cells by Granulocyte Colony Stimulating Factor (GCSF)	Pilot and I/II	Bone marrow stem cell mobilization	NCT01825551 NCT00397423 NCT00298597 NCT02236065
	OECs: olfactory ensheathing cells	Pilot	Stem cell transplantation	(L. Chen et al., 2012; Lin Chen et al., 2007; Huang et al., 2008)
	Neural progenitors	I and II	Stem cell transplantation	NCT02943850 NCT01348451 NCT01730716 NCT01640067 NCT02478450
Mitochondrial function and metabolism	Combination Product: CoQ10, vitamin E, N-Ac cysteine, and L-cystine	II	Antioxidant	NCT04244630
	Coenzyme Q10 (CoQ10)	II	Mitochondrial co-factor	NCT00243932 NCT04244630 NCT02709330
	Triheptanoin	I and II	Oxidative phosphorylation and ATP generation	NCT 03506425
	Tauroursodeoxycholic acid (TUDCA)	II and III	Antioxidant	NCT00877604 NCT03800524 NCT03127514 NCT03488524
	Tamoxifen	II	Upregulation of mitochondria respiratory chain	NCT00214110
	Creatine	II	ATP source	NCT00005674
	Creatine Monohydrate	III	ATP source	NCT00069186
RNA	Antisense oligonucleotides	I and III	RNase H1-induced of target mRNA	NCT01041222 NCT02623699

metabolism	(ASOs)		degradation	NCT03070119
	Sodium phenylbutyrate (PB)	II	Histone deacetylase inhibitor	NCT03127514 NCT03488524 NCT00107770