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Neuromuscular Junction Dysfunction in Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder characterized by progressive degeneration of motor neurons leading to skeletal muscle denervation. Earlier studies have shown that motor neuron degeneration begins in motor cortex and descends to the neuromuscular junction (NMJ) in a dying forward fashion. However, accumulating evidences support that ALS is a distal axonopathy where early pathological changes occur at the NMJ, prior to onset of clinical symptoms and propagates towards the motor neuron cell body supporting "dying back" hypothesis. Despite several evidences, series of events triggering NMJ disassembly in ALS are still obscure. Neuromuscular junction is a specialized tripartite chemical synapse which involves a well-coordinated communication among the presynaptic motor neuron, postsynaptic skeletal muscle, and terminal Schwann cells. This review provides comprehensive insight into the role of NMJ in ALS pathogenesis. We have emphasized the molecular alterations in cellular components of NMJ leading to loss of effective neuromuscular transmission in ALS. Further, we provide a preview into research involved in exploring NMJ as potential target for designing effective therapies for ALS.

Keywords Amyotrophic lateral sclerosis · Neuromuscular junction · Motor neuron · Skeletal muscle · Terminal Schwann cells · Dying back

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal progressive disorder with degeneration of corticospinal/corticobulbar (upper) motor neurons (UMN) in cerebral cortex and bulbar/spinal (lower) motor neurons (LMN) in brain stem and spinal cord. Major clinical symptoms are weakness and atrophy of limb muscles, difficulty in swallowing and speaking, and finally death due to respiratory failure. The clinical profile of ALS is heterogeneous with mean survival period of 3 to 5 years, but based on age, gender, and site of onset of symptoms, the duration of survival is variable [1–4]. While familial ALS constitutes ~ 5 to 10% in the global context, in India, however, it is much less at 1 to 2% [5]. Genetic mutations in more than 100 genes have been reported in familial ALS. Among these, mutations in SOD1, C9ORF72, TDP-43, and FUS genes are the most frequent. Many of these genes have also been implicated in sporadic ALS [6].

UMN makes direct (monosynaptic) or indirect (via interneurons) connections with LMN which innervates effector muscles in the periphery and triggers their contraction (Fig. 1a). Loss of motor neurons (MN) causes rapid weakness of voluntary muscles due to denervation and corresponding changes of neuromuscular junction (NMJ) structure. Fatigue or easy tiredness is an important symptom of ALS, and this feature was the basis for evaluation of neuromuscular transmission in the clinical setting. Neuromuscular transmission defect can be demonstrated by neurophysiology technique of repetitive nerve stimulation (RNS). After the first report by Mulder et al. [7], several studies observed that ALS patients with muscle fatigue showed a decremental response during RNS which indicates

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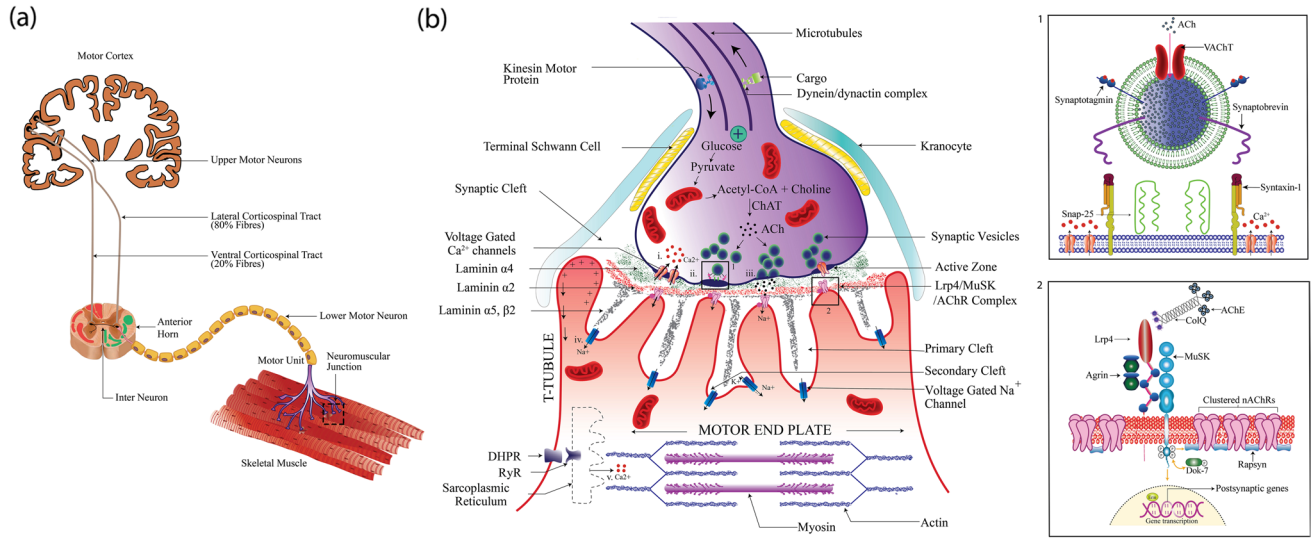

Fig. 1 Structural and functional organization of healthy motor neurons and neuromuscular junction. **a** Upper motor neurons projects from motor cortex and descends to brainstem/spinal cord via corticospinal tracts where it synapses with lower motor neurons which innervate skeletal muscles. **b** Interaction of tripartite components of neuromuscular junction: presynaptic motor neuron, postsynaptic skeletal muscle, terminal Schwann cells and capping kranocytes. Motor neuron and skeletal muscles are separated via synaptic cleft filled with basal lamina consisting of different laminins isoforms. Action potential arriving at nerve terminal triggers clustered voltage-gated Ca^{2+} channels at the active zones. This leads to (i) influx of Ca^{2+} ions, (ii) docking, (iii) fusion, and release of synaptic vesicles filled with neurotransmitter ACh into synaptic cleft. Binding of ACh to postsynaptic clustered nAChRs generates localized endplate potential (EPP). EPP triggers (iv) voltage-gated Na^{+} channels and generates muscle action potential which propagates along the muscle fiber and activates dihydropyridine receptors (DHPR) located in T-Tubules. DHPR-mediated ryanodine receptor (RyR) activation causes (v) release of Ca^{2+} from sarcoplasmic reticulum which eventually causes contraction of muscle fiber via the actin myosin contractile units. Inset 1 Organization of synaptic vesicles at active zone. Synaptotagmin, synaptobrevin, SNAP-25, and syntaxin are crucial for docking, fusion, and release of ACh into the synaptic cleft. Inset 2 Organization and signaling of postsynaptic Lrp4/MuSK/nAChRs complex

instability of neuromuscular transmission [8–10]. Further, decrease in motor unit number estimation (MUNE) in ALS patients indicated progressive loss of motor axons which affects neuron-muscle communication [11–13].

Despite advances in the field of ALS, site of disease origin still remains a key unresolved question. Initiation of neurodegeneration in ALS can be explained by the dying forward and dying backward hypotheses [14]. The dying forward hypothesis proposes that UMN drives anterograde degeneration of LMN via glutamate excitotoxicity which eventually descends to NMJ [15–17]. This hypothesis was proposed by Eisen and team in 1992 [18], and they demonstrated inverse correlation between cortical threshold and motor evoked potentials (MEP)/ compound muscle action potential (CMAP) ratio indicating cortical hyperexcitability [19]. Later, several studies supported early cortical dysfunction in ALS patients which probably arises due to altered cortical excitatory/inhibitory circuitry [15, 20–25]. Even transgenic ALS mice displayed increased intrinsic excitability and bioenergetics defects in UMN [26, 27]. Further, exposure of LMN to excitotoxin in mice caused motor neuron degeneration and NMJ abnormalities in an anterograde manner [17].

These studies raised an important question, whether excitotoxicity mediated neurodegeneration arises due to intrinsic hyperexcitability of MN? Several research groups have reported variable excitability of LMN during the course of ALS progression. At early stage, LMN from mutant SOD1 mice displayed hyperexcitability which arises due to increased persistent Na (+) current in large LMN [28–32]. However, some studies have also observed unaltered or decreased excitability of LMNs [33–35]. At later stage, most LMN displayed normal excitability despite increase in input conductance. These MN were able to achieve homeostasis for excitability by upregulating depolarizing current, whereas some MN exhibited hypoexcitability [36, 37]. The inability of LMN to fire repetitively probably arises due to homeostatic deregulation of excitability [38]. Altogether, these reports indicate that alterations in electrical properties are not caused by intrinsic excitability of MN but rather involves extrinsic factors such as synaptic activity. Some of the crucial findings supporting dying forward phenomenon have been outlined in Fig. 2a. For better understanding, readers can refer to excellent recent reviews by Brunet and Eisen [39, 40] on early cortical dysfunction.

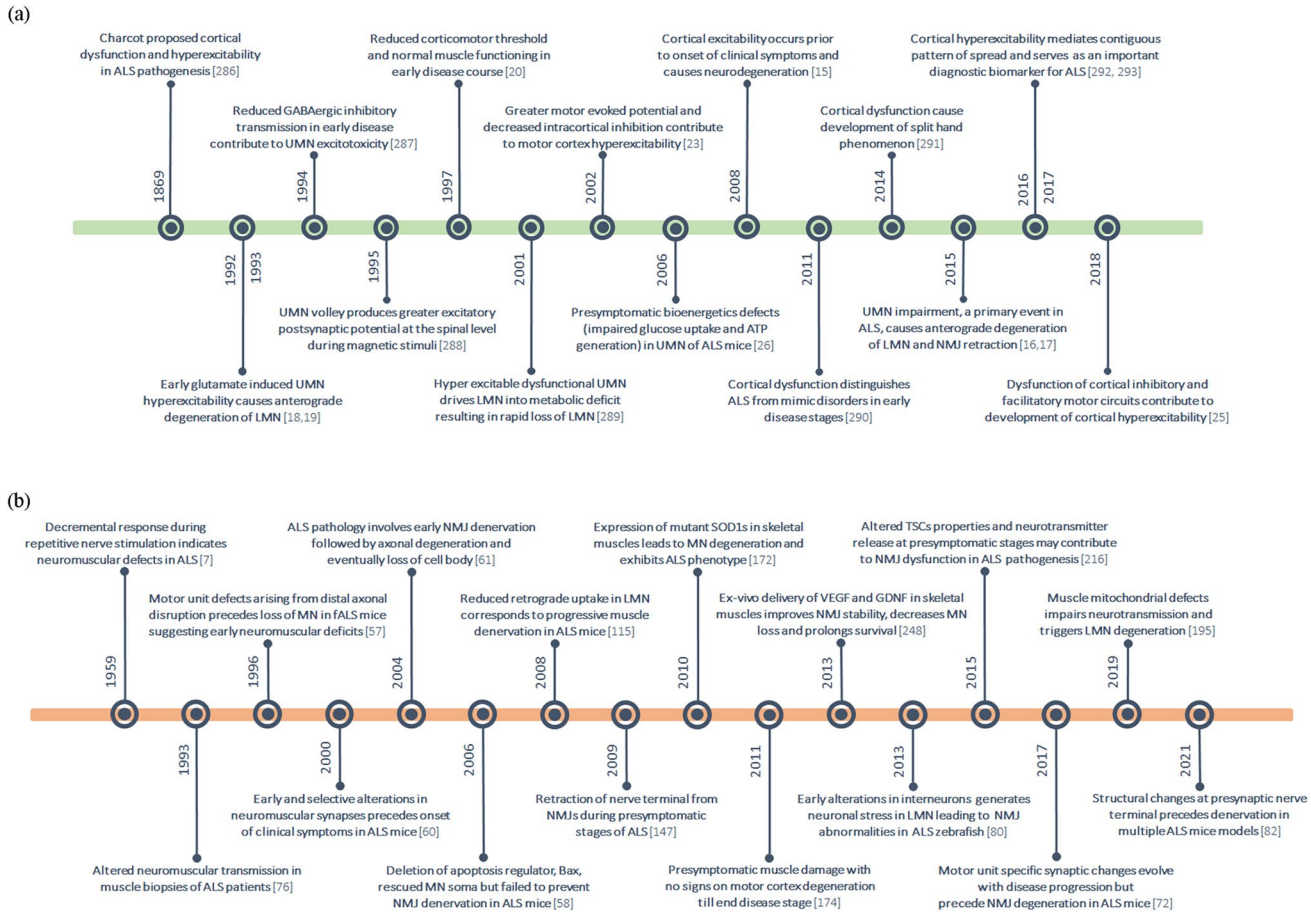

Fig. 2 Timeline of major findings supporting **a** dying forward and **b** dying backward hypothesis in ALS. UMN, upper motor neuron; LMN, lower motor neuron; VEGF, vascular endothelial growth factor; GDNF, glial cell line-derived neurotrophic factor; TSC, terminal Schwann cells

Contrary to dying forward hypothesis, the dying back hypothesis propounds that MN degeneration begins distally at the nerve terminal/neuromuscular junction and progresses towards the cell body in a retrograde fashion [41, 42]. Below, we provide a comprehensive overview of cellular and molecular perturbations at neuromuscular junction in ALS and thereby focusing on evidencing supporting the dying back hypothesis.

Neuromuscular Junction

Neuromuscular junction is a specialized chemical synapse where information in the form of electrical impulses is transmitted from motor neurons to skeletal muscles to initiate muscle contraction and execute voluntary motor functions. The NMJ is a tripartite synapse comprising of three major components, viz., presynaptic motor neurons, postsynaptic muscles, and terminal Schwann cells (Fig. 1b). Kranocytes, the fibroblast-like cells identified as the fourth component of NMJ, are responsible for capping TSCs and entire endplate area [43]. Although their role in synapse formation and functioning is not known, they appear to affect synaptic regeneration [44].

The well-coordinated reciprocal interactions among these components are essential for regulating formation and maturation of NMJ. Structural organization of pre- and postsynaptic apparatus, such as active zones on nerve terminals, clustering of nicotinic acetylcholine receptors (nAChRs) on the postsynaptic membrane, is indispensable for proper functioning of NMJ as illustrated in Fig. 1b. Active zones are electron dense multiprotein complex responsible for exocytosis of neurotransmitter. Active zones contain voltage-gated calcium channels (VGCC), soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins including syntaxin-1 and SNAP-25, and cytoskeletal matrix at the active zone (Fig. 1b inset 1). VGCCs are responsible for rapid influx of Ca^{2+} which helps in interaction of SNARE proteins and leads to docking, fusion, and release of neurotransmitter acetylcholine (ACh) into the synaptic cleft (Fig. 1b).

Postsynaptic muscle membrane contains densely clustered nAChR that directly oppose presynaptic active zones. During NMJ development, agrin released by

motor nerve terminal binds to postsynaptic lipoprotein receptor-related protein 4 (Lrp4) which activates muscle-specific receptor tyrosine kinase (MuSK) domain and leading to its self-phosphorylation [45]. Activated MuSK phosphorylates Dok-7, a muscle cytoplasmic adaptor protein, which in turn phosphorylates and further activates MuSK. Dok-7 interacts with MuSK through its phosphotyrosine-binding domain and controls its activity and responsiveness to agrin [46]. MuSK acts as a master regulator of synaptic differentiation by activating a series of downstream molecules which eventually induces dense clustering of nAChR at the postsynaptic membrane. MuSK activates ETS-related molecule (ERM) which regulates expression of various postsynaptic genes including nAChR subunits genes [47]. MuSK recruits rapsyn, an intracellular scaffolding protein which strongly interacts with nAChRs, leading to recruitment and clustering of nAChRs at the NMJ (Fig. 1b inset 2) [48]. Clustered nAChRs are anchored into the cell membrane by actin cytoskeleton and dystrophin-glycoprotein complex. Detailed cellular pathway involved in NMJ formation is reviewed in Li et al. [49].

The space between the presynaptic nerve terminal and postsynaptic muscle membrane is called synaptic cleft. Synaptic cleft spanning ~ 50 nm contains basal lamina, made up of extracellular matrix (ECM), which surrounds the muscle fibers [50]. Basal lamina contains various ECM proteins such as laminins, collagens, perlecan, and fibronectins which play a pivotal role in NMJ assembly and functioning [50, 51]. Laminins play a pivotal role in organizing pre- and postsynaptic components and thereby maintain structural integrity of NMJ. Laminin β2, secreted by muscles, interacts with presynaptic VGCC which leads to clustering of VGCC and recruitment of other presynaptic components at the active zones [52]. Laminin β2-deficient mice displayed decreased calcium sensitivity and co-localization of active zone proteins and led to alteration in motor endplate maturation and thereby suggested its role in synapse stabilization [53]. Laminin α4 and α5 interaction with dystroglycan helps in maturation of nAChR clusters which promotes postsynaptic differentiation [54]. Loss of laminin α4 disrupted alignment and stabilization of pre- and postsynaptic components at the NMJ and resulted in altered neuromuscular transmission [55].

This spatiotemporal organization of NMJ components is cardinal for efficient neuromuscular transmission which involves a series of highly complex and dynamic processes. Any alterations in organization and/or signal transmission can impair NMJ functionality leading to denervation, muscle weakness, or paralysis.

Evidences of Neuromuscular Junction Disruption as Primary Event in ALS

Most of the initial research encompassing ALS majorly revolved around MN degeneration as it was believed that ALS primarily affects MN and alterations in skeletal muscle arise as a consequence of loss of neurons. The hypothesis of distal involvement stemmed from early observations that LMN loss was much more evident than UMN [56] and further loss of axonal integrity led to dysfunctional NMJ [57]. Importantly, preserving MN by deletion of Bax, a key regulator of apoptosis, in $SOD1^{G93A}$ ALS mice successfully rescued MN from mutant SOD1-mediated toxicity but failed to prevent NMJ denervation [58]. Similarly, inhibition of p38 MAPK, a stress activated protein kinase involved in initiating cell death, exerted positive effects on MN survival but failed to rescue NMJ denervation in ALS mice [59]. These reports have highlighted crucial role of NMJ disruption in disease mechanism.

To understand the mechanism underlying NMJ dysfunction in ALS, various cellular models based on mutations in SOD1, FUS, TARDBP, and C9ORF72 genes have been exploited. Studies have reported that transgenic SOD1 ALS mice showed significant NMJ denervation followed by degeneration of ventral root axons and eventually loss of MN cell bodies indicating early loss of NMJ circuitry [60–62]. Apart from mutant SOD1, transgenic mice expressing mutant FUS experienced early structural and functional alterations at the NMJ followed by MN degeneration [63, 64]. Transgenic mice with inducible expression of mutant TDP-43 (ΔNLS, loss of nuclear localization signal) displayed significant denervation with no changes in number of LMN in spinal cord. Suppression of mutant TDP-43 in postsymptomatic stages prevented ongoing MN loss and increased NMJ re-innervation which helped in functional recovery from mutant TDP-43-mediated toxicity [65]. Similarly, inducible expression of hexanucleotide (G_4C_2) C9ORF72 repeats in mice showed dipeptide repeats expression which led to structural NMJ abnormalities and rapid muscle dystrophy with no change in number of LMN in spinal cord. Interestingly, early suppression of C9ORF72 expression was able to prevent but not reverse muscle dystrophy [66]. Mutant FUS-ALS drosophila displayed significantly impaired synaptic neuromuscular transmission with normal electrical excitability of MN cell bodies indicating early neuromuscular dysfunction [67]. Similarly, $SOD1^{G93A}$ zebrafish displayed early loss of intact NMJs including decrease in postsynaptic volume followed by loss of MN cell bodies [68, 69].

It is important to mention that there is a temporal loss of different motor units during disease progression. ALS pathology involves presymptomatic loss of NMJs of fast

fatigable (FF) motor units followed by loss of fast fatigue-resistant (FFR) motor units at symptomatic stage and eventual loss of slow (S) motor units at end-stage disease [60, 70, 71] as illustrated in Fig. 3b. High metabolic demand and large size of FF MN makes them more vulnerable to degeneration whereas small size of type S MN makes them resistant. It has recently been highlighted that reduced neurotransmitter release in NMJs of FF motor units precedes NMJ denervation affecting pre- and postsynaptic elements in $SOD1^{G37R}$ mice [72]. However, the initial denervation triggers a compensatory mechanism where the nearby neurons increase their axonal arbors to form new synaptic connection to mask ongoing NMJ dismantling [73–75]. Repeated cycles of denervation and reinnervation results into an intermittent conduction block in newly formed nerve endings, indicating that immature sprouts and unstable conduction of NMJ are a result of degenerating axons in ALS.

In humans, very few studies have identified early changes at NMJ as it is difficult to obtain presymptomatic samples. Therefore, it is challenging to validate the findings from animal model in human patients at presymptomatic disease stages. In 1993, Maselli et al. [76] reported altered synaptic transmission in muscle biopsies of ALS patients. Histological examination of NMJ in these patients showed absence of axon terminal indicating denervation and corresponding decrease in amplitudes of miniature endplate potentials as measured by electrophysiological techniques. In this study, normal postsynaptic folds were observed in limited number of denervated muscle samples analyzed. In contrast, a recent study revealed significantly altered postsynaptic apparatus and NMJ denervation with fragmented synaptic gutters in muscle samples of early and long-term ALS patients. They also observed flattened and fragmented postsynaptic apparatus being partially innervated by axon terminal indicating reinnervation and remodeling of NMJ as observed in animal models. [77]. A case report also showed severe denervation

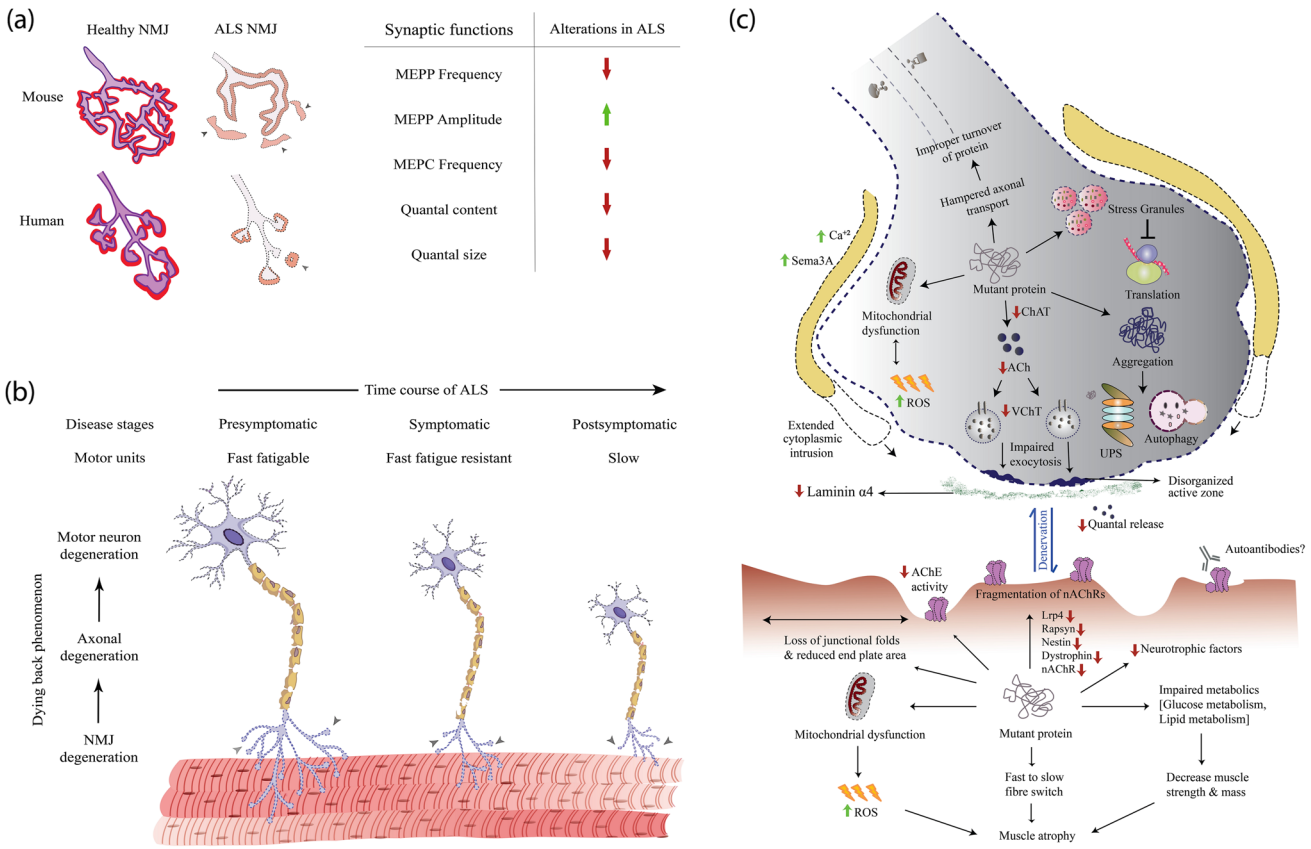

Fig. 3 Neuromuscular junction disassembly in amyotrophic lateral sclerosis. **a** Structural and functional alterations in NMJ in ALS. Left panel shows the interaction of presynaptic and postsynaptic membranes in humans and rodents in healthy and ALS condition. Presynaptic membrane is in purple for healthy and gray for ALS, whereas postsynaptic is in red for healthy and light pink for ALS. Arrow heads denote orphaned nAChRs. Right panel defines the altered functional parameters of NMJ in ALS. **b** Specificity and temporal loss of motor units during ALS progression. Arrow heads denote retraction of presynaptic nerve endings from skeletal muscle. **c** Cellular and molecular alterations in the presynaptic neuron, postsynaptic muscle, and terminal Schwann cells resulting in NMJ denervation in ALS. ALS-associated mutant proteins differentially affect the components of NMJ which trigger and/or amplify malfunctioning of NMJ in ALS

with no changes in LMN in autopsy of a 58-year-old male, who died in a minor surgery, after 6 months of onset of ALS symptoms [61]. These studies on ALS patients indicate altered NMJ integrity as an early event in disease course. It is important to note that there are considerable differences in cellular architecture of NMJ of different species. In humans, NMJs are smaller and have "nummular" morphology, whereas mouse NMJs are larger and "pretzel"-shaped. Despite these differences, evidences from human and animal models clearly supports that NMJ is compromised in early ALS stages (Fig. 3a).

Altogether, such studies corroborate that malfunctioning of NMJ may be the primary pathogenic event in ALS and MN loss proceeds in a dying back fashion. Evidences from animal and human studies supporting early role of NMJ in ALS have been summarized in Fig. 2b. In the sections below, role played by presynaptic motor neurons, postsynaptic skeletal muscle cells, and terminal Schwann cells in triggering NMJ disassembly is described to delineate the series of events culminating in neurodegeneration.

Role of Motor Neuron in NMJ Disruption in ALS

Lower motor neuron communicates with skeletal muscles and executes voluntary actions by means of neuromuscular transmission. LMN cell bodies lie within the central nervous system, i.e., brainstem and anterior horns of spinal cord from where they extend long processes to reach target muscle and form an indispensable link between CNS and skeletal muscles. Due to long axons, communication between cell body and nerve terminal is majorly dependent upon anterograde and retrograde axonal transport system. Maintaining homeostasis in cell body and distal nerve terminal is necessary for healthy functioning of NMJ. ALS-associated mutant proteins may lead to deregulation of protein homeostasis, malfunctioning of axonal transportation, and cholinergic dysfunction and impairs mitochondrial dynamics as illustrated in Fig. 3c.

Mutant Protein and Deregulated Protein Homeostasis

ALS-associated mutant proteins can cause structural and functional changes in the presynaptic nerve terminal which may trigger NMJ degeneration. Motor neuron of $SOD1^{G93A}$ mice showed the presence of enlarged and vacuolated mitochondria in axons and presynaptic nerve terminal. There was also decrease in soma size and accumulation of small, empty vacuoles in axons which results in axonal loss and muscle denervation [78]. AAV vector-mediated silencing of SOD1 in motor neurons of $SOD1^{G93A}$ mice led to delayed disease onset, preserved NMJ functionality, and prolonged survival [79]. In addition, interneurons play a key role in modulating synaptic activity. Transgenic SOD1 ALS zebrafish showed the earliest sign of neuronal stress in inhibitory glycinergic interneurons at embryonic stage. This resulted in loss of inhibitory input which generated neuronal stress in LMN. In adult life, these stressed LMN showed retracted nerve terminal resulting in NMJ abnormalities whereas non-stressed neurons have normal NMJs [80, 81]. Systematic and structural screening of NMJs in mutant FUS and TDP-43 ALS mice showed early structural changes in presynaptic nerve terminal but no corresponding changes at the motor endplate. These changes preceded denervation which led to reduced interaction between pre- and postsynaptic membrane suggesting that cues for NMJ degeneration may arise from MN terminal [82]. Overexpression of mutant FUS in motor neurons of transgenic flies resulted in reduction in number and area of presynaptic bouton which led to significant locomotive impairment [83].

The large size of LMN makes them more vulnerable to proteome imbalance which causes ER stress, mitochondrial impairment, altered excitability, and synapse dysfunction [84]. Prolonged deregulation of protein homeostasis leads to aberrant accumulation of misfolded mutant proteins such as SOD1, TDP-43, and FUS which have been implicated in ALS. Most of the mutations in SOD1 enzymes have a propensity to form high molecular weight aggregates. These mutant SOD1 aggregates have been reported in ALS patients as well as mice models [85, 86]. Ubiquitin proteasome system (UPS) was found to be impaired with increased accumulation of ubiquitin and phosphorylated neurofilaments in motor neurons of $SOD1^{G93A}$ mice [87]. Even, we have observed effect of modulation of protein homeostasis pathways on high molecular-weight aggregates of $SOD1^{L84F}$ [5] in motor neuronal cell line (unpublished work).

Expression of mutant FUS at physiological level in mice showed cytoplasmic mislocalization of mutant protein which accumulates at rough endoplasmic reticulum and alters expression of gene associated with ribosomes, mitochondria, and proteasome suggesting altered proteostasis as a potential pathomechanism of mutant FUS [88]. Mutant FUS decreases synaptic activity by downregulating expression of transporters and ion channels required for synaptic functioning in ALS mice. Moreover, accumulation of mutant FUS generates stress response and inhibits local, intra-axonal protein synthesis [89]. Mutant TDP-43 mice exhibited alterations in gene splicing, and downregulation of genes is involved in ubiquitin proteasome pathway along with ubiquitin-positive inclusions [90]. ALS-associated polydipeptide repeat expansion in C9ORF72 gene causes ribosome-mediated distress and hampers protein translation in ALS mice [91].

Degradation of improperly folded protein is mediated by ubiquitin proteasome system and autophagy. A recent study showed that cranial MN exhibit high proteasome activity making them more resistant to proteostasis stress as compared to spinal MN indicating time-dependent death of spinal and cranial MN in ALS disease course [92]. Autophagy plays a key role in maintaining cellular homeostasis in distal axons by balancing synthesis and degradation of proteins [93]. Activation and upregulation of autophagy has been observed in cytoplasm of MN in sALS patients [94]. Mutations in several genes associated with autophagy including SQSTM1, OPTN, UBQLN2, and TBK1 have been associated with ALS [95–98]. NMJs of $SOD1^{G93A}$ mice showed increased accumulation of autophagosomes in nerve terminals of MN along with reduced expression of SQSTM1 suggesting altered autophagy [99]. Spinal MN restricted expression of mutant $UBQLN2^{P497H}$ in transgenic rats recapitulated ALS-like phenotype such as MN degeneration, denervated NMJs, and abnormal protein accumulation [100]. Autophagy inhibition by Atg7 or TBK1 deletion in MN of $SOD1^{G93A}$ mice accelerated NMJ denervation accompanied by significant decrease in endplate current amplitude in tibialis anterior muscle [101, 102]. Moreover, deletion of TBK1 in TDP-43^{G298S} mice aggravated NMJ integrity [103]. These studies provide a vital link between mutant proteins, alteration in protein degradation pathways, and NMJ stability in ALS pathology.

Disturbed Axonal Transport

Axonal transportation involves neuronal cytoskeleton (microtubules, actin, and intermediate filaments) and motor proteins such as dyneins, kinesins, and myosins. Anterograde transportation of various organelles and cargos (containing mRNA, proteins, and lipids) from cell body to axon terminals as well as retrograde transportation of neurotrophic factors, signaling molecules, and damaged organelles from distal nerve terminal to cell body are crucial for functioning and survival of MNs [104]. Most of kinesin motor family proteins are involved in unidirectional anterograde transportation, whereas dynein is responsible for retrograde transportation (Fig. 1b).

Dynein is a dimeric multisubunit complex and requires an essential cofactor dynactin to mediate axonal transportation. Dynactin is a large protein complex made up of several subunits and acts as a processivity factor for dynein. Mutations in largest subunit, p150 (Glued), of dynactin complex have been reported in ALS patients [105–107]. Mutant dynactin p150 (Glued) mice displayed pathological features of motor neuron disease characterized by impaired vesicular transport, denervation, axonal swelling, and axon-terminal degeneration [108]. Moreover, decreased mRNA and protein levels of dynactin1 are observed in spinal MN of sALS patients [109, 110]. Depletion of dynactin 1 in *C. elegans* and zebrafish displayed axonal degeneration accompanied by severe motor defects, abnormal innervation of fast twitch muscle fibers, NMJ instability, and locomotor defects [111, 112].

Aberrant neurofilament aggregation is a pathological hallmark of ALS. Mutant SOD1-mediated toxicity is responsible for abnormal accumulation of neurofilaments which disrupts axonal transport and leads to axonal degeneration and profound reduction in retrograde uptake in early stages of disease [113–117]. These defects in axonal transportation has been attributed to activated p38 MAP kinase. Inhibition of p38 MAPK rescued proximal axons from SOD1-mediated toxicity and improved survival of SOD1 ALS mice [59, 118]. In addition, mutant SOD1 inhibits anterograde fast axonal transport by activating p38 MAP kinase [119]. Mutant TDP-43 impairs axonal trafficking in drosophila and MN derived from ALS patients [120]. Recently, a genome-wide association study identified mutation in C-terminal of kinesin family member 5A (KIF5A) gene in ALS patients [121, 122]. These studies highlight the role of axonal transport in maintaining distal synapse, and defects in transportation machinery may strongly impact NMJ stability.

Cholinergic Dysfunction and Altered Neurotransmission

Efficient cholinergic transmission is crucial for maintaining structural and functional integrity of NMJ. Cholinergic dysfunction is a common feature of various neurodegenerative disorders including ALS. Choline acetyltransferase (ChAT) is responsible for synthesis of ACh, patterning, and formation of neuromuscular synapses [123]. Decreased ChAT activity has been reported in spinal MN of ALS patients [124, 125]. Even $SOD1^{G93A}$ ALS mice displayed early reduced ChAT content in soma and synaptic terminals of MNs indicating that presymptomatic cholinergic dysfunction could be the earliest event in ALS [126]. Previously, SOD1 aggregates also impaired transportation of ChAT by sequestering kinesin-associated protein 3 and thereby disrupt cholinergic system in SOD1 ALS mice [127].

Vesicular ACh transporter (VAChT) is involved in packing of ACh in synaptic vesicles at nerve terminals and serves as a marker for cholinergic synapses. Immunohistochemical staining of spinal MN of ALS patients displayed decreased VAChT but no decrease in activity of synaptophysin, a marker for synapse integrity, indicating loss of cholinergic input prior to MN degeneration [128]. Interestingly, increasing synaptic ACh by overexpressing VAChT diminished motor functions and accelerated NMJ degeneration and death of $SOD1^{G93A}$ mice [129]. These observations suggest that increased or decreased levels of VAChT have detrimental effect on cholinergic transmission.

Efficient neurotransmission is quantified by measuring localized postsynaptic depolarization (endplate potential (EPP)) caused by release of synaptic vesicles. These changes in postsynaptic membrane in turn reflects the functioning of presynaptic neuron. Muscle biopsies from ALS patients revealed decreased amplitude of miniature EPP (MEPP) along with decreased mean quantal stores and EPP quantal content suggesting impaired neuron and muscle communication [76]. Prior to morphological NMJ alterations, $SOD1^{G37R}$ ALS mice showed decreased quantal content indicating presymptomatic functional changes at NMJ [72]. Similarly, mutant TDP-43 ^{Q331K} mice displayed increased MEPP amplitude, decreased MEPP frequency, and quantal content at the NMJs prior to motor neuron loss [130]. These results indicate early molecular alterations in fusion and release of synaptic vesicles from the presynaptic axon terminal.

Transgenic zebrafish harboring mutant TDP-43 or mutant FUS also showed aberrant synaptic fidelity with reduced frequency of miniature endplate currents (MEPCs) and quantal neurotransmission [131–133]. Mutant FUS ALS mice showed marked depletion in synaptic vesicles in axon terminal and reduced motor response amplitude in tibialis anterior muscles suggesting altered neurotransmission [63]. Mutant FUS transgenic flies developed presynaptic structural and function defects prior to degeneration of MN cell body and axons. Mutant flies displayed disorganized active zone protein and reduced quantal content resulting in marked decrease in postsynaptic evoked current [67]. Overexpression of mutant FUS in *C. elegans* and in motor neurons of drosophila resulted in impaired synaptic vesicle docking and reduced quantal size and quantal content demonstrating reduced neuromuscular transmission [134, 135]. In addition, silencing of C9ORF72 (C9-miR) in zebrafish resulted in NMJ abnormalities accompanied with reduced frequency of MEPC suggesting impaired presynaptic functionality. C9ORF72 has been shown to interact with SV2a, a key component of presynaptic active zones. C9-miR causes decrease in release and uptake of neurotransmitter suggesting loss of C9ORF72 affects exocytosis [136]. These studies suggest that derangements in neurotransmission (synthesis, packaging, and release of neurotransmitter) are early events in ALS pathology which may contribute to distal degeneration.

Mitochondrial Dysfunction

MNs are large, polarized cells that rely heavily on mitochondrial ATP for their high energy demands. Nerve terminals at the NMJ are packed with mitochondria which are transported from cell body to synaptic terminals by means of fast axonal transport. Synaptic mitochondria are crucially involved in ATP production, calcium handling, synthesis, and release of neurotransmitter and thereby play a critical role in neuronal transmission [137].

Deficits in mitochondrial dynamics and their axonal transport have been observed in ALS [138, 139]. Mutant SOD1 and TDP-43 transgenic mice exhibited abnormal mitochondrial morphology and transport in distal part of motor axons of phrenic or sciatic nerve before disease onset indicating that altered mitochondria may trigger cascade of events leading to MN degeneration [140, 141]. Even, FUS ALS transgenic mice showed early mitochondrial abnormalities in presynaptic MN along with decreased synaptic vesicles and NMJ area before onset of clinical symptoms [142]. Similarly, transgenic mice with regulatable mutant TDP-43 (ΔNLS) expression also displayed altered mitochondrial activity. Accumulation of mutant TDP-43 in axons and nerve terminals leads to formation of RNP granules and affects localized protein translation which in turn decreases level of key nuclear-encoded mitochondrial proteins (Altman et al. unpublished work, https://doi.org/10.21203/rs.3.rs-87662/v1).

Mutant SOD1 induces degradation of mitochondrial Rho GTPase 1 (Miro1) which inhibits mitochondrial axonal transport [143]. Miro1 is responsible for attachment of mitochondria to kinesin-1, and its activity depends on cytosolic Ca^{2+} levels. Mutations in another ALS causing gene, VAPB, increase cytosolic Ca^{2+} which effects Miro1/kinesin-1 interaction and hampers mitochondrial axonal movement [144]. NMJs of $SOD1^{G93A}$ mice showed increased number of degenerating mitochondria with large vacuoles and irregular cristae spacing in presynaptic nerve terminals. Moreover, there was decreased expression of mitophagy-related proteins SQSTM1, Pink1, Parkin, and Bnip3 indicating impaired mitophagy [99]. Double knockout mice with ablation of Pink1 and Parkin resulted in increased accumulation of mitochondria in presynaptic terminal, axon swelling, and NMJ fragmentation which highlights the key role of impaired mitophagy in NMJ degeneration in ALS [99].

Impaired mitochondrial axonal transport and mitophagy causes decrease in healthy mitochondrial pool and improper turnover of damaged mitochondria in synaptic terminals which could significantly alter NMJ dynamics. Therefore, disruption of mitochondrial integrity alters NMJ dynamics and may lead to neurodegeneration.

Role of the Skeletal Muscle in NMJ Disruption in ALS

Skeletal muscle comprises the postsynaptic component of NMJ. Multiple mechanisms, such as alterations in postsynaptic apparatus, acetylcholine esterase (AChE) abnormalities, misfolding, and clearance of mutant proteins and mitochondrial dysfunction, have been implicated for its

degeneration in ALS (Fig. 3c). The findings from various studies provide insight into the role of muscles in disease initiation and have been discussed below in-depth.

Alterations in Postsynaptic Apparatus

Structural and functional integrity of postsynaptic apparatus including clustered nicotinic acetylcholine receptors (nAChRs), muscle-specific kinase (MuSK), and low-density lipoprotein receptor-related protein 4 (Lrp4) present on skeletal muscle is crucial for stability and functioning of NMJ (Fig. 1b inset 2). Earlier, muscles of ALS patients showed denervated endplates with various degrees of postsynaptic structural changes including flattening of primary cleft but well-preserved nAChRs [145, 146]. Similar findings were also observed in ALS transgenic mice [147]. However, elegant ex vivo experiments by Palma and team showed decreased ACh affinity and abnormal expression of AChR in skeletal muscle of ALS patients [148, 149]. Recently, muscle-specific expression of $SOD1^{G93A}$ in ALS mice resulted in smaller and fragmented NMJ with dispersed endplates, decreased ramification of primary cleft, and high turnover, and fragmentation of AChRs indicating mutant SOD1 affects AChR clustering and NMJ stability [150]. In addition, FUS ALS mice showed reduced endplate area in newborn mutants. Even impaired endplate maturation was observed in iPSC-derived myotubes from FUS-ALS patients [64]. siRNA-mediated knockdown of FUS in C2C12 cell line decreased gene expression of nAChR subunits. Similar results were obtained in $FUS^{\Delta NLS/\Delta NLS}$ and $FUS^{\Delta NLS/+}$ mice [64]. Moreover, knockdown of TDP-43 in zebrafish resulted in denervation and fragmentation of nAChR cluster [151].

These studies show that AChRs can be targeted for ALS pharmacological therapy. In fact, riluzole, the first FDA-approved drug for ALS, has been shown to block postsynaptic AChRs and may affect its function in denervated muscle fibers of ALS patients, but its biological significance still remains elusive [152, 153].

In addition to AChRs, alterations in other key postsynaptic proteins have also been documented in ALS. Lrp4 protein is essential for structural and functional integrity of NMJ. Loss of Lrp4 led to fragmented AChR clusters, reduced synaptic vesicles and junctional folds, and impaired neuromuscular transmission [154]. $SOD1^{G93A}$ transgenic mice also showed loss of postsynaptic structural proteins such as Lrp4, nestin, rapsyn, and dystrophin from NMJ before complete disassembly of AChRs [62]. Furthermore, neonatal rat muscle injected with cerebrospinal fluid (CSF) from ALS patients displayed structural damage and fragmentation of NMJ along with decreased rapsyn and increased calpain expression. However, no change in levels of AChRs protein was observed [155].

MuSK is another crucial postsynaptic protein involved in efficient functioning of NMJ. Increasing MuSK expression or stimulating MuSK via agonist antibody has been shown to reduce muscle denervation, delay in disease onset, and improve motor function in ALS mice [156, 157]. In contrast, another study showed that MuSK agonist antibody is not sufficient for preservation of motor neuron function and survival of ALS mice [158]. These opposing findings raise concern regarding MuSK activation as therapeutic for ALS.

Several studies have targeted Dok-7, a muscle cytoplasmic protein, for enhanced activation of MuSK. Muscle-specific overexpression of Dok-7 in mice increased pre- and postsynaptic area and enhanced neuromuscular transmission in diaphragm muscle [159] as previously observed for neuromuscular disorders [160]. Recently, Dok-7 gene therapy in $SOD1^{G93A}$ mice protected NMJ from degeneration, suppressed muscle atrophy, improved locomotor activity, and prolonged life span [161].

Acetylcholine Esterase Activity in ALS

One of the primary roles of AChE is termination of neuromuscular transmission by degradation of ACh at the NMJ. AChE is also believed to play non-enzymatic roles such as regulating fate of AChRs, NMJ formation, and survival. AChE knockout mice ($AChE^{-/-}$) exhibited reduced AChR cluster density, shallow and irregular junctional folds, and fragmented nerve terminals [162]. Initial studies have identified decreased AChE activity in muscle biopsies and increased AChE activity in plasma and serum of ALS patients [163–165]. Muscle biopsies of ALS patients also revealed alterations in various isoforms of AChE which resulted in decreased AChE activity in motor endplates [146, 166]. Loss of function of TDP-43 in zebrafish resulted in decreased expression of AChE leading to its decreased activity in skeletal muscles [151]. Earlier clinical trials with AChE inhibitors displayed no therapeutic effects in ALS patients whereas downregulating AChE expression using anti-sense nucleotide in presymptomatic ALS mice exerted beneficial effects by reducing MN loss and increasing its survival [167, 168]. Abnormalities in AChE seem to play an important role in ALS but more studies are needed to elucidate its pathomechanism.

Mutant Protein Toxicity

Studies have demonstrated that MN-specific expression of mutant SOD1 in transgenic mice is not sufficient to produce ALS phenotype [169, 170] whereas muscle-specific expression of mutant SOD1 is sufficient to develop ALS phenotype [171–173]. Transgenic mice with muscle restricted expression of $SOD1^{G93A}$ caused accumulation of reactive oxygen species (ROS) leading to structural and functional

aberration in muscles [171]. Later on, expression of mutant SOD in skeletal muscles was shown to cause NMJ abnormalities, distal axonopathy with swollen or vacuolated axon terminals, and loss of MN in spinal cord [172]. NMJ abnormalities included loss of presynaptic synaptophysin and postsynaptic nAChRs and rapsyn [173]. NMJ disruption and retrograde degeneration of MN may involve target deprivation of neurotrophic factors. Altogether, these studies strongly proposed the crucial involvement of skeletal muscles in ALS pathogenesis and challenge the well-established dogma that muscle atrophy occurs as a consequence of MN degeneration and supports dying back phenomenon.

A longitudinal MRI analysis of $SOD1^{G93A}$ mice showed significant reduction in muscle volume followed by progressive muscle atrophy prior to onset of clinical symptoms indicating skeletal muscle could be the primary target of mutant SOD1-mediated toxicity [174]. Mutant SOD1 causes early changes in muscle metabolic properties by altering glucose metabolism and fiber type composition and increasing lipid catabolism [175].

Turner et al. [176] reported the presence of intracellular aggregates of mutant SOD1 in skeletal muscles of ALS mice. On the other hand, Onesto and colleagues showed that enhanced proteasome activity and autophagy in murine myoblast cell line (C2C12) efficiently removed mutant SOD1 aggregates whereas these aggregates impaired proteasome activity in motor neuronal cell line (NSC-34) indicating mutant SOD1 differentially affects MN and skeletal muscles [177]. Even in ALS mice, mutant SOD1 did not form aggregates in skeletal muscles throughout the disease course, possibly due to enhanced proteasomal activity [178]. These recent studies highlight that muscle cells are more efficient in managing mutant SOD1 aggregates as compared to MN, and it has been suggested that mutant SOD1-mediated toxicity in muscle may involve a different mechanism [179].

Interestingly, Wei et al. [180] showed that despite the absence of insoluble mutant SOD1 aggregates in muscles, skeletal muscle proteins are sensitive to misfolding similar to spinal cord proteins. Moreover, they observed lower steady-state levels of heat shock proteins in skeletal muscles as compared to spinal cord in $SOD1^{G93A}$ mice and postulated that differential expression of molecular chaperones makes skeletal muscles proteins more vulnerable to misfolding, which probably explains early degeneration of muscles in ALS pathogenesis [180]. In addition, the two major protein degradation pathways showed a time-dependent activation in skeletal muscles during disease progression: ubiquitin–proteasome degradation system (UPS) is activated during early symptomatic stages, whereas autophagy upregulation is observed in presymptomatic and end stages of disease [181].

Another important observation showed that mutant SOD1-associated toxicity in muscles can be rescued by decreasing mRNA and protein levels of SOD1 using antisense oligonucleotides. The findings clearly demonstrated that lowering the expression of mutant SOD1 can maintain neuromuscular innervation, preserve compound muscle action potential, restore neuronal dysfunction, and prolong the life of ALS transgenic mice [182].

Similar to SOD1, hyper-phosphorylated TDP-43 aggregates also plays a pathogenic role in central nervous system in ALS, but their role in skeletal muscles is unclear. Previous study reported the absence of phosphorylated TDP-43 (pTDP-43) aggregates in quadriceps muscles [183], whereas recent studies have identified dense filamentous aggregates of pTDP-43 in diaphragm and axial muscles of ALS patients [184, 185]. These authors suggest that the opposing findings could arise due to muscle group-specific difference in muscle pathology. Further studies are warranted to better understand TDP-43 pathology in skeletal muscles.

Mitochondrial Dysfunction

Mitochondrial dysfunction in muscle plays a pivotal role in ALS pathogenesis and contributes to rapid disease progression [186, 187]. Numerous studies revealed abnormalities in morphology, quantity, membrane potential, and disposition of skeletal muscle mitochondria in ALS [150, 171, 188–192]. Mitochondrial dysfunction is also responsible for hypermetabolism, which is a common feature of ALS [193, 194]. Mitochondrial abnormalities are often accompanied by defective mitochondrial respiratory chain complex and increased oxidative stress. Mice with mutation in CHCHD10, a mitochondrial protein, resulted in severe mitochondrial defects in skeletal muscles which led to NMJ abnormalities including hyper-fragmentation of motor endplate. Loss of MN in spinal cord was evident in end disease stages indicating abnormalities in muscle mitochondria can trigger MN degeneration in a dying back fashion [195]. $SOD1^{G93A}$ mice showed tremendous increase in muscle mitochondrial ROS production accompanied by muscle atrophy [196, 197]. Recently, it has been shown that administration of cerebrospinal fluid from sALS patients into muscles of neonatal rats caused mitochondrial abnormalities and increased oxidative stress in skeletal muscles [155]. An in vitro model system developed by differentiating iPSCs from C9ORF72 ALS patients into skeletal myocytes also showed altered mitochondrial gene expression and oxidative stress [192].

Muscle mitochondria are crucially involved in maintaining NMJ integrity. Overexpression of mitochondrial uncoupling protein 1(UCP1) in muscles significantly affected NMJ stability and led to motor neuron degeneration. Moreover, heterozygous (mutant SOD1/UCP1) transgenic ALS mice exhibited shortened progression from onset and survival as compared to homozygous mutant SOD1 ALS mice [198].

Overexpression of mutant SOD1 in skeletal muscle of normal mice also led to defective mitochondrial dynamics by forming aggregates in muscle mitochondria without causing MN degeneration, indicating that ALS-associated muscle pathology is an early event in disease course [199].

Interestingly, mitochondrial functionality can be rescued by reducing oxidative-mediated damage. Treating $SOD1^{G93A}$ mice with Trolox, a potent antioxidant, inhibited ROS and rescued mitochondrial function which in turn stabilized NMJ turnover and complexity [88]. In addition, increased mitochondrial biogenesis by overexpression of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PGC-1α), in skeletal muscle of $SOD1^{G37R}$ mice, maintained mitochondrial activity throughout disease course which improved muscle endurance, delayed muscle atrophy without improving lifespan [200]. Taken together, the above studies highlight the involvement of muscle mitochondrial abnormalities in ALS.

Autoantibodies Against Postsynaptic Proteins in ALS

Interestingly, autoantibodies against various postsynaptic proteins have been reported in ALS. The presence of anti-acetylcholine receptor antibodies, a hallmark feature of myasthenia gravis, has been observed in ALS patients with no history of myasthenia gravis or exposure to snake's venom [201, 202]. Similarly, antibodies against Lrp4 have also been reported in peripheral blood of 9.8% American, ~ 15% Israeli, ~ 23% in Greek and Italian, and 5.4% in Chinese ALS patients [203–206]. Some studies have also reported the presence of autoantibodies against AChE in serum of ALS patients whereas others failed to observe the same [207, 208]. Although these are rare events but it raises an important question, whether autoimmune component is involved in ALS? These autoantibodies may be involved in denervation process and appear to be an important aspect to explore their role in disease mechanism.

Role of Terminal Schwann Cells in NMJ Disruption in ALS

Unlike well-characterized nerve-muscle interaction, terminal Schwann cells (TSCs) are important but overlooked component of tripartite synapse. TSCs play an imperative role in NMJ formation, maturation, maintenance, and regeneration [209, 210]. TSCs can sense and modulate synaptic activity to ensure appropriate neuromuscular transmission. Due to the close association, TSCs have a bidirectional relationship with motor nerve terminals in regulating synaptic output. Release of neurotransmitter from nerve terminal activates TSC's muscarinic AChRs (mAChRs) which causes intracellular release of Ca^{2+} from internal stores of TSCs [211, 212]. In response, TSCs release gliotransmitter such as ATP which acts on presynaptic A_1 (inhibitory) and A_{2A} (excitatory) adenosine receptors and fine-tunes acetylcholine release from the nerve terminal [213]. It has been demonstrated that degenerating nerve terminals release signaling molecules which causes TSC to acquire a macrophage like behavior. The acquired phagocytic activity helps TSCs to engulf degenerating nerve terminal, assist nerve regeneration [214], and induces new AChR clustering and helps in neuromuscular junction remodeling [215].

Studies based on ALS transgenic mice showed involvement of TSCs abnormalities in ALS (Fig. 3c). Altered Ca^{2+} signaling accompanied by higher mAChR-mediated TSC activation led to major synaptic alterations [216]. NMJs of fast medial gastrocnemius muscle lacked TSC cell bodies followed by an increased denervation as compared to NMJs of slow soleus muscle [217]. Previously, TSCs showed increased expression of Sema3A, an axon repellent molecule, in NMJs of FF type IIb/x muscle fibers which highlights the early loss of FF motor units observed in ALS pathology [218]. Another study demonstrated increased level of adenosine at the synaptic cleft during symptomatic phase suggesting impaired release of TSC gliotransmitter [219].

Importantly, muscle samples from ALS patients also exhibited altered TSC morphology with extensive cytoplasmic processes. It was observed that in certain NMJs, TSC processes invaded synaptic cleft leaving limited accessible space for synaptic neuromuscular transmission [77]. Owing to the dynamic role played by TSC in regulating NMJ homeostasis, its altered function may exacerbate ALS phenotype.

Is Targeting NMJ a Possible Treatment for ALS?

Currently, riluzole and edaravone are the only two FDA-approved drugs for ALS treatment. Edaravone, a potent antioxidant, scavenges oxygen radicals, whereas riluzole exerts inhibitory effects on glutamate release [220]. Both drugs display modest benefits in prolonged survival and/or function by alleviating symptoms and slowing disease progression [221].

As several evidences discussed above support that NMJ impairment is an early event in ALS pathology, therapeutic treatments aimed at preserving NMJs may play an imperative role in fighting against ALS. Globally, the major focus of research has been to investigate role of neurotrophic factors, microRNAs, and small molecules to preserve NMJ integrity (Fig. 4).

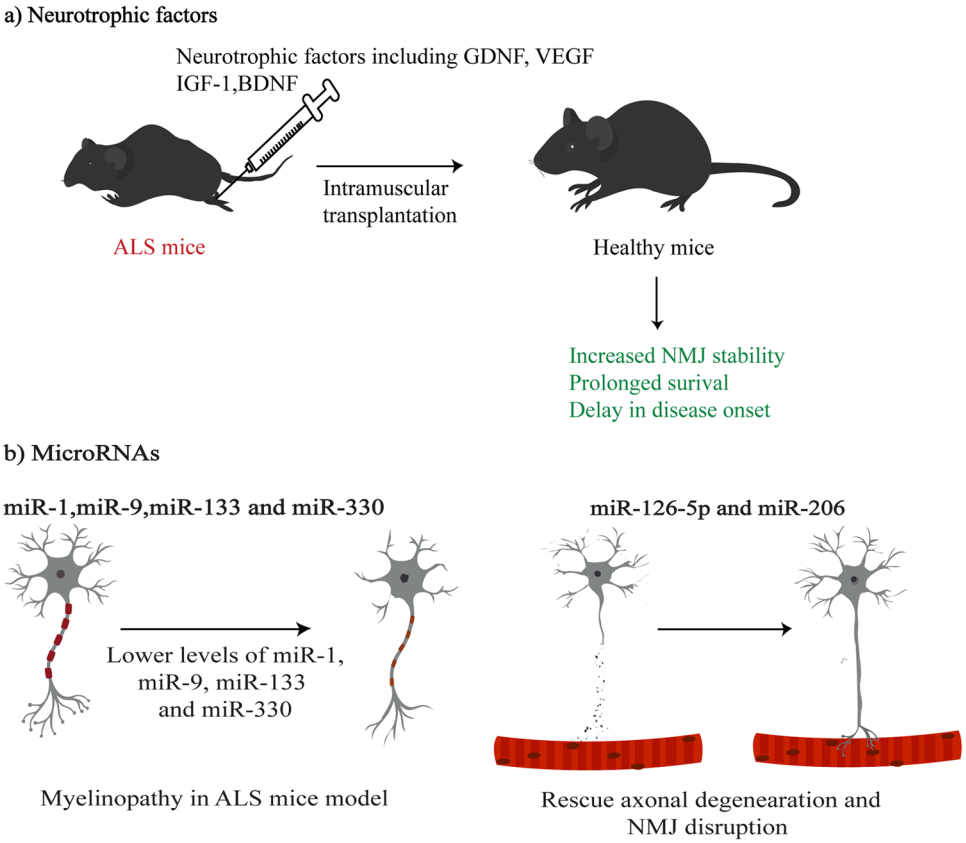

Fig. 4 Rescuing potential of neurotrophic factors and miRNAs in preserving NMJ integrity in ALS. **a** Intramuscular transplantation of neurotrophic factors helps preserve NMJ integrity and improves life span of ALS mice. **b** Manipulating muscle-specific miRNA expression rescues MN degeneration and NMJ disruption

Neurotrophic Factors

Neurotrophic factors are endogenous soluble signaling proteins which promote growth and survival of neurons. Skeletal muscle is a rich source of neurotrophic factors such as glial-derived neurotrophic factor (GDNF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and brain-derived neurotrophic factor (BDNF) which are crucial for NMJ stability, MN survival, and axonal growth. Neurotrophic factors are internalized by axon terminals at NMJ and retrogradely transported to cell bodies of MN. Alterations in expression of muscle-derived neurotrophic factors influence NMJ denervation and spinal MN degeneration.

a) *Glial Derived Neurotrophic Factor*

GDNF is a potential neuronal growth factor which plays pivotal role in presynaptic branching and hyperinnervation [222]. Exogenous GDNF treatment in healthy mice resulted in enhanced axonal branching and arborization at the NMJ in early post-natal days suggesting GNDF plays a key role in synaptic maintenance and remodeling [223]. Therefore, it is important to evaluate its role during NMJ denervation in ALS. Increased GDNF mRNA levels were observed in muscle biopsies of alive ALS patients, whereas muscle biopsies from post mortem patient samples showed decreased expression of GDNF [224, 225]. These observations indicate GDNF expression varies drastically with disease progression.

Three independent studies observed that increasing expression of GDNF in skeletal muscle of $SOD1^{G93A}$ mice resulted in significant delay in disease onset, improved locomotor performance, and prolonged survival [226–228]. Interestingly, delivery of GDNF secreting neural progenitor cells (NPCs) in spinal cords remarkably preserved MN degeneration but failed to preserve NMJ denervation resulting in no beneficial effect on limb function [229]. However, when GDNF secreting mesenchymal stem cells (MSCs) were injected in skeletal muscles, an apparent increase in NMJ connections, survival of spinal MNs delayed disease progression [230]. These findings indicate that survival of motor neurons and innervation of muscle fibers are independent events. GDNF, secreted by muscle and terminal Schwann cells, binds to Ret tyrosine kinase receptor localized at the nerve terminals of presynaptic region and promotes synapse maturation [231]. On the other hand, it has been suggested that binding of GDNF to GPI-linked coreceptors and Ret tyrosine kinase receptors on motor neurons increases its survival by activating PI3-kinase signaling pathway [232, 233]. Therefore, combined therapy aimed at targeting MN cell perikarya and NMJ

would help reduce disease progressing and alleviate ALS symptoms.

b) ***Transforming Growth Factor-Beta***

TGF-β, a motoneuron survival factor, is involved in the development and maintenance of NMJ. TGF-β is localized at the synaptic region of muscle fibers, and its receptors are distributed along the length of motor axons and nerve terminals [234]. Schwann cells also express TGF-β and promote synapse formation at NMJ. TGF-βs belong to a small family of multi-functional cytokines, consisting of three isoforms: TGF-β1, β2, and β3. A significant increase in all three isoforms of TGF-β has been observed in human and mouse ALS muscle [235]. Circulating levels (serum, plasma, and CSF) of TGF-β1 have been found to be significantly enhanced in ALS patients [236–239]. TGF-β1 activation has also been shown in both spinal cord and muscle of ALS mice [240]. In skeletal muscle of symptomatic $SOD1^{G93A}$ mice, TGF-β-mediated signaling is enhanced as compared to presymptomatic mice [241]. It has been hypothesized that reduced TGF-β signaling at early stage induces glutamate excitotoxicity and prevents its neuroprotective effect. And at the later stage, increased TGF-β levels activate microglia and hamper NMJ [242]. Another study showed increased levels of TGF-β1 at NMJs of even presymptomatic SOD1 ALS mice which repressed FGFBP1 expression and modulated NMJ innervation [243].

Interestingly, intraperitoneal injection of TGF-β2 in SOD1 mice with resulted in enhancement of motor performance without preventing motor neuronal degeneration [244]. Though the mechanistic action was not defined, but TGF-β2 has been shown to locally regulate neurotransmission by increasing the quantal content (presynaptic vesicles) [245]. All these evidences suggest that TGF-β pathway plays a role in ALS pathogenesis, but detailed analysis to understand the fine balance between levels of different TGF-β isoforms and downstream targets is important to explore its role in diagnostics and therapeutics.

iii) ***Vascular Endothelial Growth Factor***

VEGF exerts its protective effects by decreasing astrogliosis and increasing NMJ formation in ALS mouse [246]. Lentiviral expression of VEGF in skeletal muscles of ALS mice protected MN degeneration, slowed disease onset and progression, and enhanced life expectancy by 30% [247]. Intramuscular transplantation of MSCs expressing GDNF and VEFG improved lifespan, NMJ stability, and decreased MN loss in $SOD1^{G93A}$ transgenic rats [248].

iv) ***Insulin-Like Growth Factor-1***

IGF-1 plays a key role in neuronal survival and maintenance of neuromuscular connections. Significantly reduced serum levels of IGF-1 were observed in ALS patients [249]. Even skeletal muscle samples from ALS patients showed decreased levels of IGF-1 and IGF binding proteins [250]. However, a recent study reported increased serum IGF-1 levels in ALS patients with longer survival indicating higher IGF-1 levels may be involved in delaying disease progression [251].

Muscle-specific expression of IGF-1 in ALS mice attenuated muscle atrophy and displayed well-preserved AChR clusters and stabilized innervation of muscle fibers. Moreover, increased IGF-1 led to prolonged MN function and life span [252, 253]. IGF-1 injected in skeletal muscle of ALS mice was retrogradely internalized by MN which protected mitochondria from autophagy by reducing cytochrome C release and inhibited apoptosis [254].

e) ***Brain-Derived Neurotrophic Factor***

BDNF is involved in maintaining structural integrity and synaptic function in adult muscular synapses by binding to tropomyosin-related kinase B receptor (TrkB) [255]. Nerve-induced muscle contraction regulates BDNF/TrkB signaling which in turn affects release of ACh by modulating presynaptic protein kinases PKC and PKA [256]. Earlier studies have reported alterations in BDNF expression and TrkB signaling in spinal cord and muscle tissues of ALS patients [257–259]. A recent study identified C270T polymorphism in BDNF as susceptibility locus in Han Chinese ALS patients [260].

Impaired BDNF/TrkB signaling was observed in NMJs of plantaris muscle in symptomatic $SOD1^{G93A}$ mice [261]. Exercise regime modulated BDNF/TrkB signaling resulting in reduced MN death [262]. Interestingly, in vitro incubation with ALS-CSF downregulated BDNF expression and induced neurodegeneration in NSC-34 motor neuronal cell line [263, 264] and exogenous supplementation of BDNF-ameliorated ALS-CSF-induced neurodegeneration [264]. Even intrathecal administration of ALS-CSF led to significant downregulation of BDNF mRNA levels in spinal cord and upregulation in extensor digitorum longus muscle of neonatal rats [155, 265]. The increased muscle BDNF expression could be a compensatory mechanism to restore homeostasis, but due to increased NMJ denervation, MNs are not able to harness the enhanced neurotrophic levels.

MicroRNAs

MicroRNAs (miRNAs) are non-coding, single-stranded RNA molecules involved in post-transcriptional regulation of gene expression. miRNAs typically bind to 3′ untranslated region (3′UTR) of messenger RNAs (mRNAs) and lead to translational repression or mRNA degradation. Recent evidences suggest that deregulated expression of miRNAs can contribute to motor neuron disorders [266, 267]. miRNAs are key mediators for development of skeletal muscle and

nervous system. They have also been implicated in maturation, maintenance, and repair of NMJ. Altered levels of miRNA expression have been reported in motor neurons and skeletal muscles of ALS patients [268, 269]. Moreover, mutations in FUS, TDP-43, and SOD1 have been shown to reduce miRNA biogenesis in MN by inhibiting DICER catalytic activity [269]. Thus, it is important to evaluate the role of these non-coding RNAs in ALS pathology.

a) ***miR-1 and miR-133***

miR-1, a conserved muscle-specific miRNA, has been shown to contribute to NMJ stability. miR-1 and miR-133 mediate differentiation and proliferation of skeletal muscle by regulating histone deacetylase 4 (HDAC4, a transcriptional repressor of muscle gene expression) and serum response factor, respectively [270, 271].

Lower levels of miR-1, miR-133a, miR-133b, and miR-206 were observed in ALS patients undergoing physical rehabilitation which shows that miRNA expression is altered during skeletal muscle recovery in ALS patients [272].

Muscle-specific expression of $SOD1^{G93A}$ has been shown to affect spinal cord miRNA. The levels of miR-1, miR-9, miR-133, and miR-330 were reduced in ALS transgenic mice. These miRNAs are involved in activation of genes linked with myelination events in spinal cord and upregulate expression of peripheral myelin protein 22 and myelin protein zero [273]. In contrast, miR-133b was found to be upregulated following denervation in ALS mice [274].

b) ***miR-126-5p***

miR-126-5p has been shown to regulate ALS-associated genes, like VEGF-A, agrin, and C9orf72. In presymptomatic ALS mice model, lower levels of miR-126-5p induced overexpression of Sema3A and other destabilizing factors in skeletal muscles and its coreceptor Neuropilin 1 in axons. This triggered NMJ disruption and axon degeneration [275]. At the same time, overexpressed miR-126-5p transiently rescued axonal degeneration and NMJ disruption.

iii) ***miR-206***

miR-206, a skeletal muscle-specific microRNA, has been shown to promote maintenance and repair of NMJ [274]. In $SOD1^{G93A}$ mice, knocking out miR-206 enhanced disease progression and accelerated skeletal muscle atrophy. Elevated expression of miR-206 in $SOD1^{G93A}$ mice after nerve damage promoted reinnervation at NMJ by regulating HDAC4 and fibroblast growth factor pathway, thereby delaying disease progression [276]. Further, levels of miR-206 were found to be upregulated in plasma and muscles of $SOD1^{G93A}$ mice and serum of ALS patients suggesting its possible role in ALS pathogenesis [277].

iv) ***Other miRs***

In skeletal muscle of ALS patients, miR-23a, miR-29b, miR-206, miR-455, and miR-31 were found to be significantly upregulated. It was demonstrated that miR-23a binds 3′UTR of PGC-1α mRNA, a regulator of mitochondrial biogenesis, and suppresses protein translation, thereby impairing skeletal muscle mitochondrial function [268]. On the contrary, miR-9 and miR-124 were downregulated in MN of ALS patients [269].

Taken together, these evidences suggest that miRNAs are key elements responsible for regeneration and repair of NMJ, proliferation, and differentiation of skeletal muscles. However, the role of miRNAs in disruption of NMJ in ALS etiology remains to be elucidated.

Small Molecules

Various therapeutic compounds including neuroleptics are being explored for stabilizing NMJ in ALS. Pimozide, T-type Ca^{2+} channel blocker, has been shown to stabilize synaptic transmission in *C. elegans*, zebrafish, and mice model of ALS. Further, pimozide displayed its beneficial effects on NMJ function in a 6-week long phase II randomized controlled trial on ALS patients [278]. On the contrary, Pozzi et al. [279] reported chronic administration of pimozide exerted no beneficial effect on muscle strength performance but rather resulted in diminished motor performance and exacerbated NMJ loss in $SOD1^{G93A}$ and $TDP\text{-}43^{A315T}$ ALS mice. Pimozide is undergoing a long-term clinical trial to determine its effectiveness in slowing ALS progression (ClinicalTrials.gov Identifier: NCT03272503). Recently, TRVA242, a derivative of pimozide, substantially rescued locomotor deficits, NMJ structural abnormalities, and restored NMJ transmission in *C. elegans*, zebrafish, and mice model of ALS [280]. Although TRVA242 showed promising results, further studies evaluating pharmacodynamics, pharmacokinetics, behavioral effects, and other measures are required.

Conclusion

ALS, primarily considered as a motor neuron disorder, is now being viewed as a non-cell autonomous disease which involves interplay of neuronal and non-neuronal cell populations which act together to exacerbate the disease [281]. Non-neuronal cell populations such as glial cells, skeletal muscles, and peripheral blood mononuclear cells play a key role in ALS pathophysiology [171, 282–284]. Despite leaps of advancement in the field of ALS, one of the key unresolved issues is site of disease initiation. Considering the complex etiology of ALS, it is postulated that MN degeneration involves multifactorial origin and can progress in either a “dying forward” or a “dying backward” manner. There is

an ongoing debate regarding cortical hyperexcitability or neuromuscular junction as the site of ALS onset. Evidences supporting both the hypothesis have been reviewed, but we have focused more on the dying back hypothesis.

Research in the past 2 decades have identified neuromuscular junction alterations in early disease course. This review presents a plethora of clinical and experimental evidences describing cellular and molecular alterations in motor neurons, skeletal muscles, and terminal Schwann cells in ALS. MN in ALS display disturbed anterograde and retrograde axonal transport leading to insufficient maintenance of distal terminals affecting cholinergic transmission and mitochondrial dynamics as well as poor uptake of neurotrophic factors. Impaired protein homeostasis and proteins aggregation have been shown to trigger NMJ denervation. Similar to MNs, skeletal muscles are also involved in NMJ malfunctioning. Muscle-specific expression of ALS-associated protein is able to generate ALS phenotype indicating causal role of skeletal muscle in disease pathogenesis [171, 172]. Alterations in postsynaptic structures such as AChRs, Lrp4, and AChE lead to endplate dispersion, fragmentation of AChRs, decreased ramification of junctional folds, and impaired neuromuscular transmission in ALS. Interestingly, autoantibodies against these postsynaptic structures have been reported in ALS, but their pathological significance is still elusive. Unlike MNs, muscle cells have a robust proteasome machinery due to which ALS-associated mutant proteins fail to accumulate in muscles. These deleterious proteins cause mitochondrial dysfunction and alters muscle metabolic properties which leads to hypermetabolism in ALS. Decreasing mutant SOD1 expression or reducing oxidative stress have been shown to rescue mitochondrial function, restore neuronal dysfunction, and stabilize NMJ complexity [150, 182]. Terminal Schwann cells, a less explored component of NMJ, plays a pivotal role during NMJ reinnervation. TSCs abnormalities such as altered Ca^{2+} signaling, morphology, and irregular release of gliotransmitter, axon repellant molecule can impair NMJ maintenance and regenerative capabilities of TSCs. Apart from these tripartite components, basal lamina also plays a critical role in structural and functional organization of NMJ. Limb muscle samples from ALS patients showed absence of laminins α4 from NMJs [285].

The current therapies targeting motor neurons have not been very effective against ALS, and therefore, new targets for developing effective therapies for ALS are being explored. Experimental strategies aimed at preserving NMJ by increasing muscle expression of neurotrophic factors have shown to exert beneficial effects such as preserving innervation, enhancing MN survival, delaying disease onset, and improving lifespan. Similarly modulating miRNAs expression rescues NMJ denervation and aids in alleviating ALS symptoms.

Although recent studies have highlighted early NMJ pathomechanism in ALS, information regarding the series of events leading to NMJ denervation are scattered. It is still unclear whether NMJ denervation is a MN or muscle induced event? In order to find answer to such questions, extensive research is required to closely observe early pathological events involved in disease initiation. In vivo studies focusing on monitoring various aspects of NMJ during presymptomatic stages will help in better understanding of cellular and molecular changes occurring in early disease stages. Similarly, in vitro studies exploring co-culture of motor neuron, skeletal muscles, and TSCs could provide deep insights into NMJ formation, functioning, and maintenance in ALS. Furthermore, time-dependent studies comparing NMJs of vulnerable (limb muscles) and resistant (extraocular muscles) motor units in ALS will enhance our understanding of altered synaptic functionality in ALS. Altogether, such studies will provide a better understanding of early pathological changes at NMJ and help in connecting the missing dots that will unravel the mystery behind ALS.

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