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A molecular systems architecture of neuromuscular junction in amyotrophic lateral sclerosis

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A molecular systems architecture is presented for the neuromuscular junction (NMJ) in order to provide a framework for organizing complexity of biomolecular interactions in amyotrophic lateral sclerosis (ALS) using a systematic literature review process. ALS is a fatal motor neuron disease characterized by progressive degeneration of the upper and lower motor neurons that supply voluntary muscles. The neuromuscular junction contains cells such as upper and lower motor neurons, skeletal muscle cells, astrocytes, microglia, Schwann cells, and endothelial cells, which are implicated in pathogenesis of ALS. This molecular systems architecture provides a multi-layered understanding of the intra- and inter-cellular interactions in the ALS neuromuscular junction microenvironment, and may be utilized for target identification, discovery of single and combination therapeutics, and clinical strategies to treat ALS.

Over the past 50 years, nearly 40,000 studies have been conducted on amyotrophic lateral sclerosis (ALS)[1]. These studies have largely taken a necessary but reductionist approach to understand the parts of ALS - a complex systems disease. This study aims to inspire the field to embrace systems biology approaches to advance the ALS research. Specifically, this study hypothesizes that the development of a molecular systems architecture of the neuromuscular junction (NMJ) microenvironment in ALS may: 1) enable the visualization of complex biomolecular systems interactions across the NMJ microenvironment involving seven cell types; 2) reveal the biological processes and the underlying molecular pathways leading to disease pathogenesis; 3) identify potential targets for treatment; and, 4) provide a framework to develop predictive and quantitative models for drug development.

Systems biology reveals living organisms are comprised of dynamic networks of biochemical reactions wherein disease is characterized by the disruption of one or more signaling cascades that lead to gain or loss of specific biological function. The current drug development paradigm, focused primarily on reductionist approaches, has significant weaknesses: inability to develop combination therapies[2], lack of personalization "one-size-fits-all," inability to integrate the complexity of molecular interactions, and lack of a robust computational framework to predict systems-level adverse effects[3]. It is hypothesized that a systems biology approach may offer a new paradigm for drug development to address these weaknesses[4].

In this study, we aim to employ a systems biology approach to the NMJ microenvironment in ALS. Specifically, a systematic review of the current understanding of NMJ microenvironment in ALS is performed to develop a *molecular systems architecture* i.e., a visual multi-layered representation of the interactome of molecular interactions within and across motor neurons, glial cells, skeletal muscle cells, Schwann cells, endothelial cells, and oligodendrocytes that are part of the NMJ microenvironment. In ALS, the anatomical components of the NMJ microenvironment, as shown in Fig. 1, are necessary for such a development. The insights from this study aim to provide the ALS research community with a visual systems-level understanding of the complexity of biomolecular interactions involved in ALS pathogenesis, potential targets leading to ALS pathogenesis, crosstalk across the NMJ microenvironment, and a framework for computational modeling to develop new therapies.

In complex diseases, networked systems of molecular pathways span multiple organs, cell types, organelles, and compartments. The authors of this work, in previous studies, have applied a systems biology approach to develop a methodology that enables the identification, aggregation, and integration of the signaling cascades at the cellular to develop molecular systems architectures. Such molecular systems architectures have been successfully developed to reveal systems-level understanding of neurovascular disease[5], acute myeloid leukemia[6], periodontal disease[7], C1 metabolism in plants[8], low grade chronic inflammation[9], and liver detoxification[10]. The resulting molecular systems architectures have provided a framework

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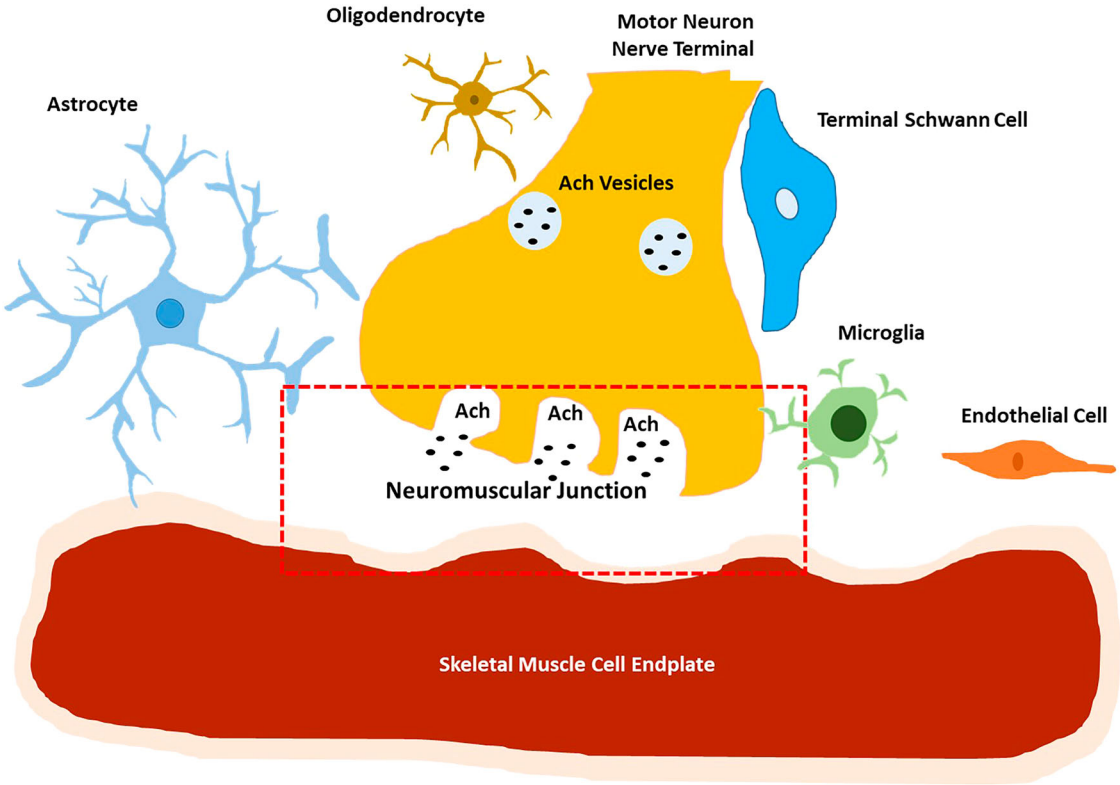


Fig. 1 | The Neuromuscular Junction (NMJ) Microenvironment. NMJ is the region where signal transduction for muscle contraction occurs by the release of acetylcholine (Ach) from the motor neuron terminal to the skeletal muscle cell endplate. The NMJ microenvironment includes astrocytes, oligodendrocytes, terminal Schwann cells, microglial cell, and endothelial cells. In ALS, dysfunction in signal transduction across NMJ leads to denervation of the skeletal muscle cell, muscle atrophy, and neurodegeneration. Ach Acetylcholine.

for computational models that provide in silico quantitative predictions to support in vitro, in vivo, and clinical research.

ALS is a complex, multifactorial systems disease[11]. ALS patients commonly experience impairment in the peripheral nervous system, particularly in the NMJ. This impairment has been show to manifest at the NMJ in the form of denervation of NMJs[12], reduction in the size of the endplate[13], and dysfunction of terminal Schwann cells (SCs)[14]. Given the heterogeneity and complexity of ALS pathogenesis, only a limited number of treatments are available. So far, only four drugs have been approved by the Federal Drug Administration (FDA) that have only a moderate effect on arresting the disease progression[3], and may have several adverse effects.

ALS is a fatal motor neuron disease characterized by progressive degeneration of both the upper motor neurons, which are found in the cerebral cortex as well as the lower motor neurons, which are found in the medulla and anterior horn of the spinal cord that supply voluntary muscles[15]. The progression of ALS usually begins in the limbs (usually the arms in two-thirds of patients) or in the bulbar muscles responsible for speech and swallowing, and then spreads to adjacent muscles: respiratory muscles, myotomes, etc.[16]. Survival for ALS patients ranges from 2 to 5 years from the onset of symptoms[17]. Broadly, ALS is categorized into two types: 1) sporadic ALS (sALS) that affects 85% to 95% of patients; and, 2) familial ALS (fALS), having mutations in known ALS genes that affects the remaining population[18].

ALS pathophysiology is characterized by involvement of multiple processes including ER stress, glutamate excitotoxicity, neuroinflammation, dysfunction in protein metabolism, inhibition of the ubiquitin-proteasome system, dysfunction of autophagy and mitophagy, and disorders in proteins involved in axonal transport[19–21]. The disease also involves alterations at the level of RNA processing with the appearance of aberrant and toxic RNAs that lose their functions and formation of pathological protein clusters in the neuronal cytoplasm[22]. Increasing evidence suggests that the gut microbiota can influence both the central and peripheral nervous systems thereby contributing to the ALS pathology[23]. Metagenomic sequencing of the gut microbiome has identified distinct microbial compositions in individuals with ALS compared to healthy individuals. ALS patients exhibit higher levels of *B. pseudocatenulatum, B. bacteria*, *A. hadrus*, and slightly lower levels of *E. coli* and *C. leptum*[24].

In this stydy, it is hypothesized that the development of a molecular systems architecture of the NMJ microenvironment in ALS will provide the following:

1. multi-layered visual map of biomolecular interactions in the NMJ microenvironment.
2. understanding of the complex crosstalk in the NMJ microenvironment.
3. potential targets for drug development.
4. framework to develop computational models providing quantitative predictions.

Results

The systematic bioinformatics analysis resulted in the molecular systems architecture of ALS pathogenesis represented in: 1) a multi-layered systems architecture schematic; 2) a systems architecture interactome of the molecular pathways; and, 3) subsystems of the interactome.

Multi-layered schematic of the molecular systems architecture

There are four layers to the molecular systems architecture schematic of ALS pathogenesis as shown in Fig. 2.

The Trigger layer consists of genetic and environmental factors. The most commonly mutated genes are summarized in Table 1. Among these genes, superoxide dismutase 1 (SOD1), Trans-activation response DNA

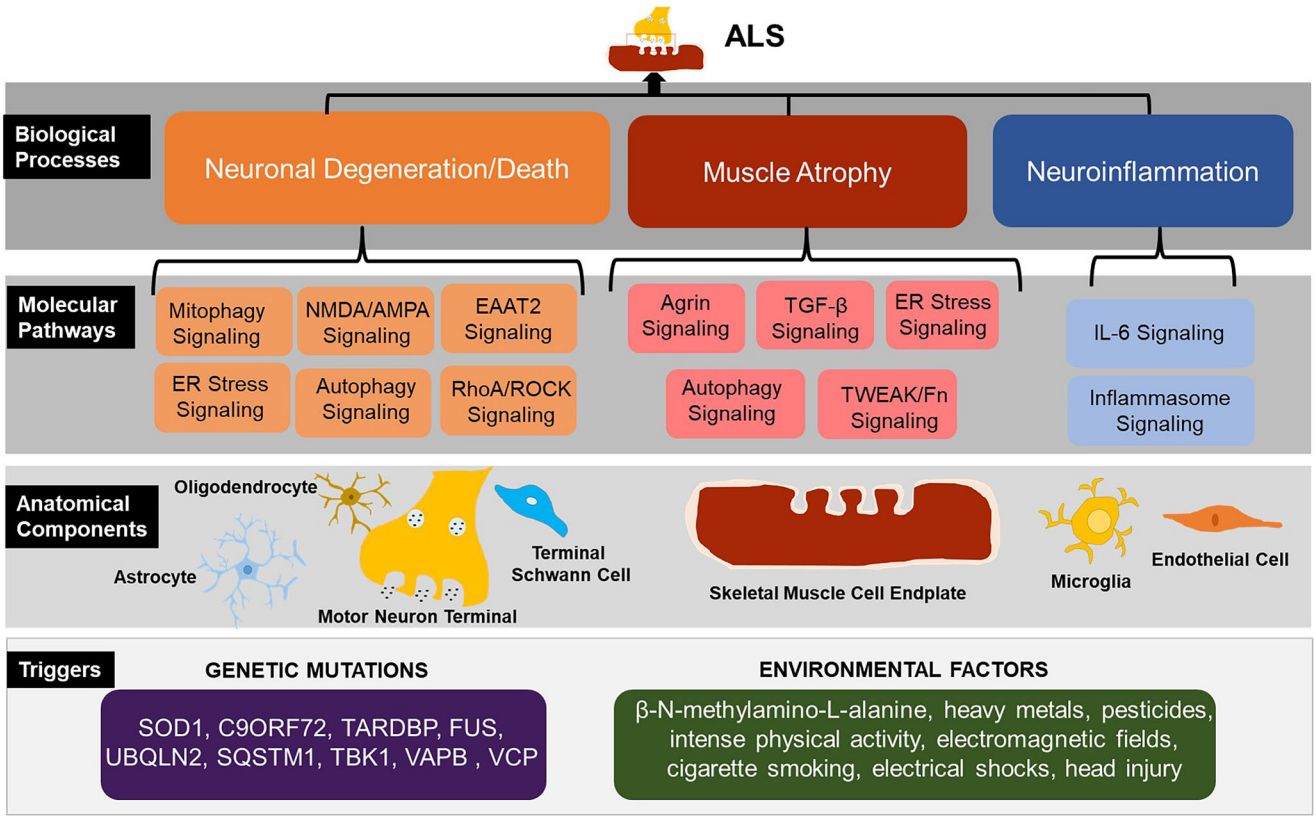


Fig. 2 | Molecular systems architecture of neuromuscular junction (NMJ) microenvironment signaling in ALS. In the four-layered architecture, the bottom layer, Triggers, consists of potential triggers: genetic mutations and environmental factors that are implicated in affecting the anatomical components leading to dysfunction in molecular pathways of ALS pathogenesis. The second layer from the bottom, Anatomical Components, consists of the anatomical components of the NMJ: astrocytes, oligodendrocytes, motor neuron terminal, terminal Schwann cell, skeletal muscle endplate, microglia, and endothelial cells that are involved in the pathogenesis of ALS. The third layer from the bottom, Molecular Pathways, consists of the molecular pathways within and among the anatomical components. The top layer, Biological Processes, reveals the three biological processes: neuronal degeneration/death, muscle atrophy, and neuroinflammation, resulting from the dysfunction in molecular pathways of the NMJ microenvironment, leading to ALS. EAAT Excitatory amino acid transporter; NMDA N-methyl D-aspartate, AMPA α-amino-3-hydroxy- 5-methyl-4-isoxazole propionic acid; RhoA Rat sarcoma homolog family member A, ROCK Rho-associated protein kinase, TGF-β Transforming growth factor beta, ER – Endoplasmic reticulum, IL-6 Interleukin-6, Tweak Tumor necrosis factor-like weak inducer of apoptosis, Fn14 Fibroblast growth factor-inducible 14, SOD1 Superoxide dismutase 1, C9ORF72 Chromosome 9 open reading frame 72, TARDBP Trans-activation response DNA binding protein, FUS Fused in sarcoma, UBQLN2 Ubiquilin-2, SQSTM1 Sequestosome 1, TBK1 TANK Binding Kinase 1, VAPB Vesicle-associated membrane protein-associated protein B/C, VCP Valosin-containing protein.

binding protein 43 (TDPBP), fused in sarcoma (FUS), and chromosome 9 open reading frame 72 (C9orf72) manifest in over one-half of fALS patients and in up to 10% of sALS patients. Specifically, SOD1, FUS and TARDBP have been directly associated with NMJ dysfunction[25]. Rare mutations of other genes have been associated with ALS including ubiquilin-2 (UBQLN2), sequestosome 1 (SQSTM1), TANK Binding Kinase 1 (TBK1), vesicle-associated membrane protein-associated protein B/C (VAPB), and valosin-containing protein (VCP)[25].

Exposure to environmental pollutants interact with individual genetic susceptibility to affect the pathogenesis of ALS. Potential environmental risk factors for ALS include exposure to head injury, cigarette smoking, cyanobacteria, heavy metals, early testosterone exposure, pesticides, intense physical activity, statin treatment, electromagnetic fields, and electrical shocks[26,27].

The second layer from bottom, as shown in Fig. 2, represents cellular components of the neuromuscular junction: motor neuron, muscle cell, astrocyte, microglia, Schwann cells, oligodendrocytes, and endothelial cells[28]. Interactions among these seven cell types occur across eleven molecular pathways that are implicated in NMJ dysfunction of three biological processes leading to ALS.

The third layer from the bottom, as shown in Fig. 2, reveals eleven molecular pathways that represent the crosstalk across the NMJ microenvironment. Dysfunction in these molecular pathways leads to biological processes causing ALS pathogenesis. These eleven pathways include: ER stress signaling, mitophagy signaling, agrin signaling, tumor necrosis factor-like weak inducer of apoptosis (Tweak)/ fibroblast growth factor-inducible 14 (Fn14) signaling, N-methyl D-aspartate (NMDA)/α-amino-3-hydroxy- 5-methyl-4-isoxazole propionic acid (AMPA) signaling, excitatory amino acid transporter (EAAT2) signaling, ras homolog family member A (RhoA)/ Rho-associated protein kinase (ROCK) signaling, transforming growth factor beta (TGF-β) signaling, autophagy signaling, interleukin-6 (IL-6) signaling, and inflammasome signaling.

The top layer, as shown in Fig. 2, identifies the three biological processes of ALS pathogenesis: neuronal death, muscle atrophy and neuroinflammation. Neuronal degeneration/death is affected by ER stress signaling, mitophagy signaling, Tweak/Fn signaling, NMDA/AMPA signaling, EAAT2 signaling, autophagy signaling, and RhoA/ROCK signaling. Muscle atrophy is affected by agrin signaling, autophagy signaling, ER stress signaling, and TGF-β signaling. Neuroinflammation is affected by IL-6 signaling and inflammasome signaling. The biological processes of neuronal death, muscle atrophy and neuroinflammation, individually and in combination, driven by the molecular subsystems identified in the Molecular Pathways layer, give rise to ALS.

Table 1 | Gene mutations in ALS

Gene Mutation	Cell Type	Associated Pathology	Reference
SOD1	Motor neurons, skeletal muscle cells	Abnormal aggregation of misfolded SOD1 protein, mitochondrial dysfunction, NMJ denervation	106
C9orf72	Motor neurons	Impaired autophagy, impaired release of quantal synaptic vesicles, MN loss, muscle atrophy, tau-mediated neurotoxicity	107
FUS	Motor neurons	Leak into cytoplasm causing DNA damage response (DDR), hyper-phosphorylation and aggregation of Tau, neuronal death	108
TDP43	Motor neurons, astrocytes	Cytoplasmic aggregation, impaired autophagy, mitochondrial dysfunction, MN death	109
Sigma 1 R	Motor neurons	Aggregation of cytoplasmic σ1R that impair its ability to bind with IP3R. This results in the loss of function in controlling calcium ion flux, activation of calpain, reduced ATP synthesis, and collapse of the mitochondrial-associated membrane	110
NEDL1	Motor neurons	NEDL1 alters the cellular localization of TDP-43 causing it to primarily localize in the cytoplasm. NEDL1 overexpression induces cellular stress resulting in cell death	111
DJ-1	Astrocytes	DJ-1 deficiency is linked to various mitochondrial morphological and functional characteristics such as fragmented mitochondria, decreased calcium absorption, compromised respiration, and modified membrane potential	112
TBK1	Motor neurons	Impaired autophagy, increased SOD1 aggregation	113
UBQLN2	Motor neurons	Hindered the capacity to transport ubiquitinated substrates to the proteasome. The disruption of proteolysis through the ubiquitin-proteasome system is likely a contributing factor to the development of ALS caused by mutant UBQLN2	114
SPTLC1	Motor neurons	Higher levels of sphinganine and ceramides accumulation	115
VCP	Motor neurons, skeletal muscle cells	Accumulation of autophagosomes and hampering the fusion with lysosomes, impaired autophagosome degradation	116
CHCHD10	Motor neurons, skeletal muscle cells	Fragmentation of the motor end plate, OXPHOS deficiency in muscle	117
UNC13A	Motor neurons	The depletion of UNC13A results in compromised neurotransmission	118
SARM1	Motor neurons	The mutations in SARM1 decrease NMNAT2 activity leading to polyneuropathy in both humans and model organisms. Absence of NMNAT2, the primary axonal isoform of NMNAT, is a protein essential for the survival of axons	119

SOD1 Superoxide dismutase 1, *C9ORF72* chromosome 9 open reading frame 72, *TDP43* Trans-activation response DNA binding protein 43, *Sigma 1 R* Sigma 1 receptor, *NEDL1* neuronal homologous to E6AP carboxyl terminus (HECT)-type ubiquitin-protein isopeptide ligase, *DJ-1* protein deglycase, *DJ-1* FUS Fused in sarcoma, *TBK1* TANK Binding Kinase 1, *UBQLN* Ubiquilin-2, *SPTLC1* Serine palmitoyltransferase, long chain base subunit 1, VCP Valosin-containing protein, *CHCHD10* Coiled-coil-helix-coiled-coil-helix domain-containing protein 10, *UNC13A* Unc-13 homolog A, *SARM1* Sterile alpha and toll/interleukin receptor motif-containing 1.

Interactome of the molecular systems architecture

Cells in the NMJ microenvironment interact with each other via a myriad of molecular pathways. These molecular interactions – the interactome as shown in Fig. 3 – occur across the motor neurons, the microglia, astrocytes, oligodendrocytes, terminal Schwann cells or skeletal muscle cells. Figure 3 provides details of the some of the key complex molecular interactions, underlying the Molecular Pathways layer in Fig. 2, that affect the biological processes of neuronal degeneration/death, muscle atrophy, and neuroinflammation identified in the Biological Processes layer of Fig. 2. The legend for the symbols in Figs. 1–15 are provided in the Supplementary Table 1.

Interactome Subsystems of Molecular Systems Architecture

The details of the interactome are provided in the Supplementary Information, which contains the seven subsystems: DJ-1 signaling (Supplementary Fig. 1); MMP-9 signaling (Supplementary Fig. 2); RIPK/MLKL signaling (Supplementary Fig. 3); NADPH oxidase signaling (Supplementary Fig. 4); SARM1 signaling (Supplementary Fig. 5); Sema3A signaling (Supplementary Fig. 6); and, Nrf2 signaling (Supplementary Fig. 7).

Crosstalk signaling across cellular components of neuromuscular junction

The molecular systems architecture provides a visual map of the multilayered molecular interactions. In addition, the molecular systems architecture integrates key recent findings revealing crosstalk across multiple cell types in NMJ microenvironment. The following are the crosstalk in signaling pathways between motor neurons and skeletal muscle cells.

The endoplasmic reticulum (ER) is a significant organelle responsible for protein quality control and maintaining cellular homeostasis[29]. ER stress signaling is a hallmark of ALS pathogenesis and is triggered by accumulation of misfolded mutant proteins such as SOD1, TDP43, FUS, C9orf72 and others, and disturbances in calcium balance triggering the unfolded protein response (UPR) in NMJ cells, particularly motor neurons and skeletal muscle cells[20,30]. Chronic activation of ER stress has been shown to cause neuronal death via UPR pathway[20]. ER stress pathways in muscle cells and motor neurons are illustrated in Figs. 4 and 5, respectively.

Overexpression mutant TDP-43 in ALS has been shown to increase CCAAT/enhancer binding proteins homologous protein (CHOP) mRNA levels leading to neuronal cell death[31]. CHOP acts as a transcription factor for activation of B-cell leukemia/lymphoma 2 (Bcl-2) interacting mediator of cell death (Bim) that facilitates TDP-43 mediated neuronal cell death[32]. Mutant TDP-43 significantly increases CHOP expression compared to wild type TDP-43[33], indicating that TDP-43 accumulation can cause ER stress leading to TDP-43-induced neurotoxicity. Additionally, ER stress caused by TDP-43 also upregulates of inositol-requiring enzyme type 1 (IRE1)/X-box binding protein 1 (XBP1) signaling pathways[34]. When overexpressed, IRE1 inhibits production of anti-apoptotic microRNAs and upregulates pro-apoptotic proteins leading to cell death[35].

Mutant SOD1 promotes ER stress by binding with Derlin-1 in the ER and hindering the movement of misfolded proteins from the ER to the cytosol. This interaction between mutant SOD1 and Derlin1 causes activation of IRE1/apoptosis signal-regulating kinase 1 (ASK1) signaling and subsequently neuronal cell death[36].

Phosphorylated TDP-43 aggregates have been found in skeletal muscles[37] and are responsible for the ER stress in the skeletal muscle cell. Misfolded protein aggregates induce ER stress and initiate UPR pathway leading to elevation of ER stress biomarkers such as PERK and eIF2α. In the

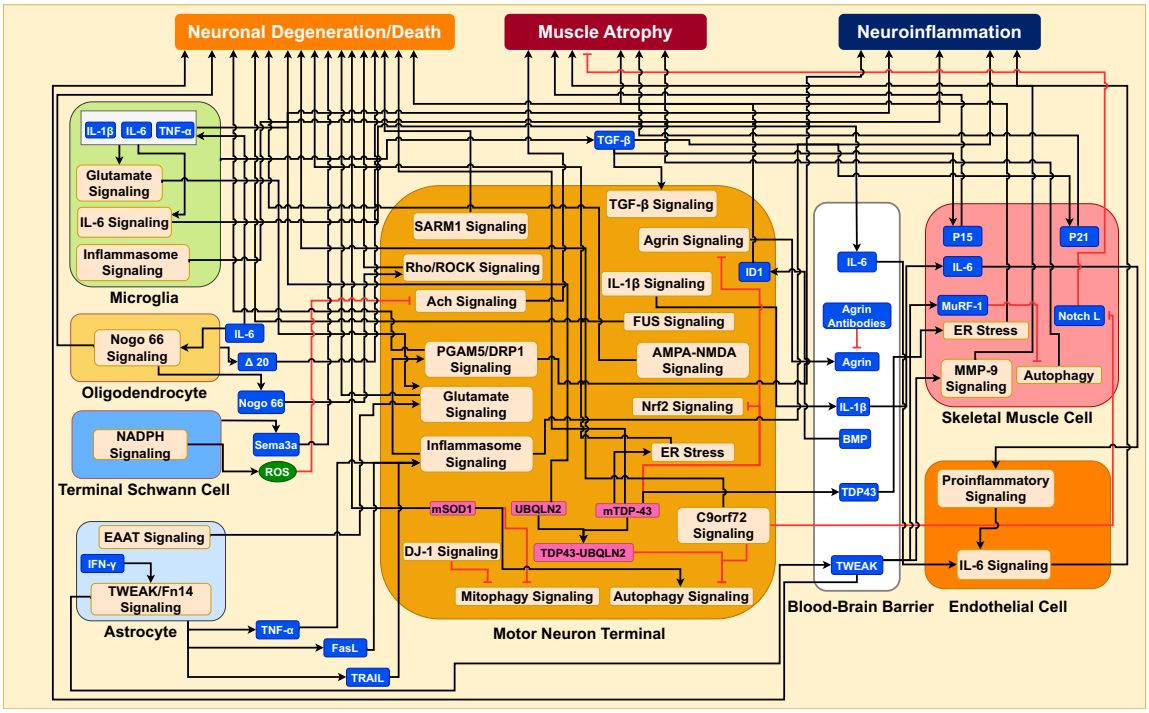
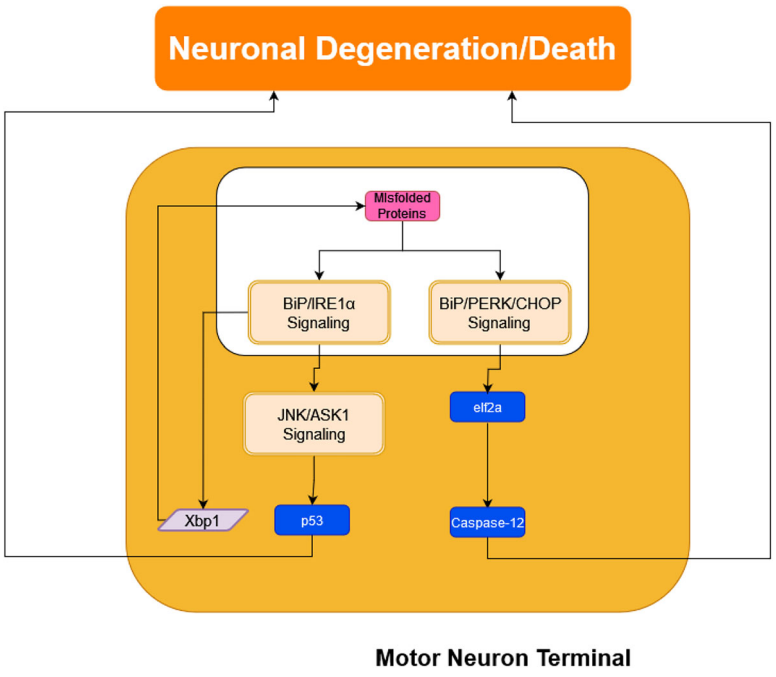


Fig. 3 | Interactome of the molecular systems architecture of the NMJ microenvironment involved in ALS pathogenesis. Molecular pathways interact across the NMJ as well as across multiple cell types: motor neuron, Schwann cell, oligodendrocyte, microglia, astrocyte, endothelial cell, and skeletal muscle. Large rounded rectangle represents a cell; double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; small dark blue rectangle represents a protein or enzyme; small pink rectangle represents mutated protein/enzyme; dark green oval represents small molecules. IL-1β Interleukin 1 β, IL-6 Interleukin 6, TNF-α Tumor necrosis factor α, NLRP3 nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3, ATP Adenosine triphosphate, ROS Reactive oxygen species, TGF-β Transforming growth factor beta, SARM1 Sterile alpha and TIR motif containing 1, Rho Rat sarcoma homolog family member, ROCK Rho-associated protein kinase, NADPH nicotinamide adenine dinucleotide phosphate hydrogen, ID1 Inhibitor of differentiation or DNA binding-1, BMP Bone morphogenetic protein, MuSK Muscle-specific kinase, ER Endoplasmic reticulum, TWEAK Tumor necrosis factor-like weak inducer of apoptosis, Fn14 Fibroblast growth factor-inducible 14, MMP-9 Matrixmetalloprotease 9, TDP-43 Trans-activation response DNA binding protein 43, Ach Acetylcholine, NMDA N-methyl D-aspartate, AMPA α-amino-3-hydroxy- 5-methyl-4-isoxazole propionic acid, FUS Fused in sarcoma, Nrf2 Nuclear factor erythroid 2-related factor 2, PGAM5 Phosphoglycerate mutase family member 5, DRP1 Dynamin related protein 1, SOD1 Superoxide dismutase 1, UBQLN2 Ubiquilin-2, EAAT2 Excitatory amino acid transporter, DJ-1 Protein deglycase DJ-1, C9orf72 Chromosome 9 open reading frame 72, MuRF-1 Muscle Ring-Finger Protein-1.

Fig. 4 | ER stress signaling leading to neuronal apoptosis. Misfolded protein initiate BiP/IRE1α signaling and BiP/PERK/CHOP signaling in the ER leading to neuronal death mediated by p53 and caspase-12. IRE1 Inositol-requiring enzyme type 1, XBP1 X-box binding protein 1, BiP Binding immunoglobulin protein, CHOP CCAAT/enhancer binding proteins homologous protein, PERK Protein kinase R (PKR)-like endoplasmic reticulum kinase; eukaryotic initiation factor-2α (eIF2α), JNK c-Jun N-terminal kinase, ASK1 Apoptosis signal-regulating kinase 1, p53 Tumor protein P53. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation, small dark blue rectangle represents a protein or enzyme; purple lozenge represents mRNA; and, small pink rectangle represents mutated protein/enzyme.



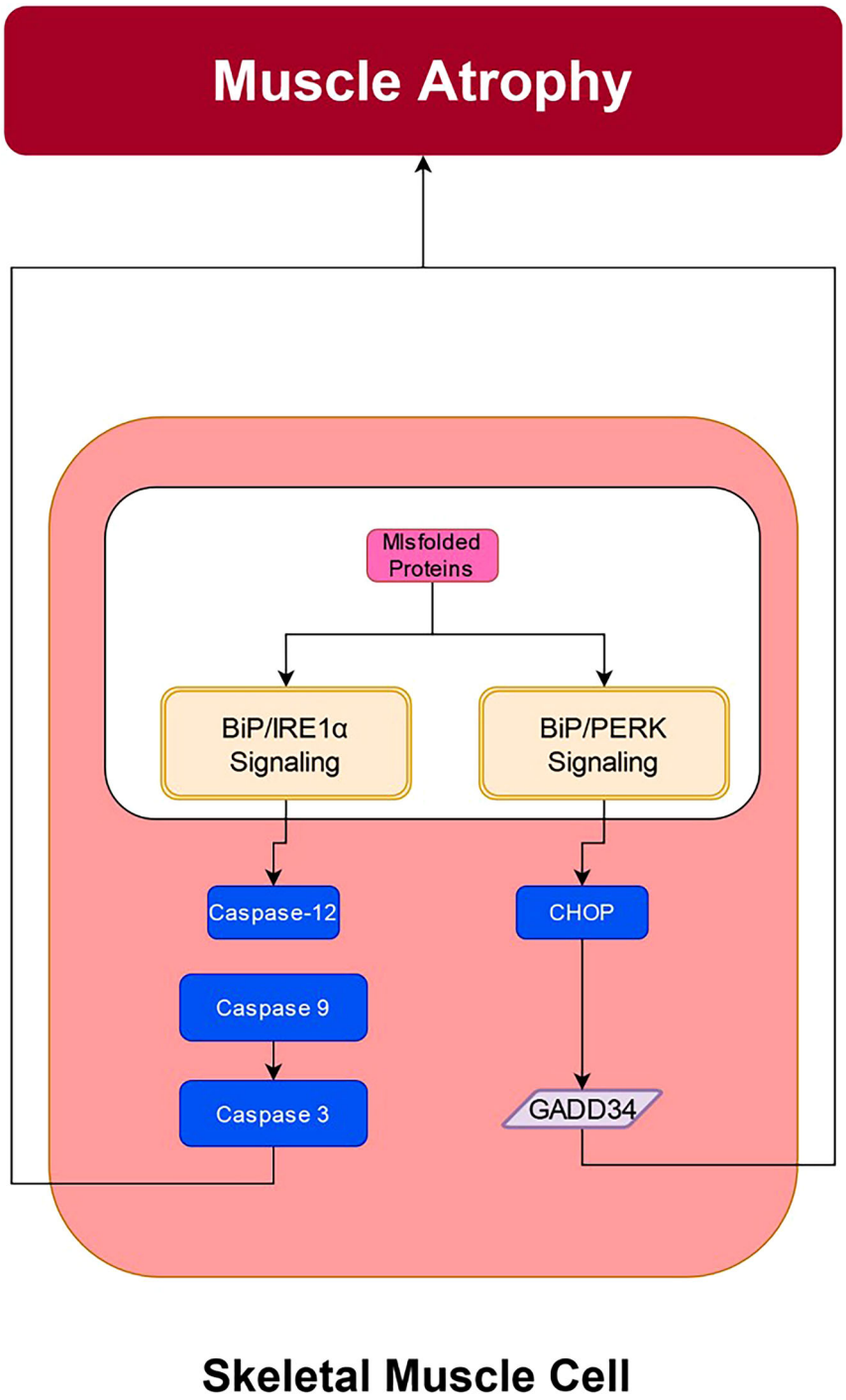


Fig. 5 | ER stress signaling in skeletal muscle cells leading to muscle atrophy. Misfolded proteins in the ER upregulate BiP/IRE1α signaling and BiP/PERK signaling in the skeletal muscle cell leading to muscle atrophy via caspase 3 and GADD34. GADD34 Growth arrest and DNA damage-inducible protein 34. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; purple lozenge represents mRNA; small dark blue rectangle represents a protein or enzyme; purple lozenge represents mRNA; and, small pink rectangle represents mutated protein/enzyme.

ER, Bip promotes the activation of PERK, which phosphorylates eukaryotic initiation factor-2α (eIF2α) leading to expression of caspase12[38]. Activation of caspase 12 triggers the apoptosis of skeletal muscle cells[39]. Phosphorylation of also PERK and eIF2α also activates CHOP, which in turn causes muscular atrophy[40].

The neurotransmitter acetylcholine (ACh), released by motor nerve terminals of the motor neurons, activates ACh receptors (AChRs) on skeletal muscle fibers leading to muscle contraction[41]. The agrin-LRP4-MuSK complex regulates the clustering of postsynaptic AChRs[42]. Agrin from nerve terminals stimulates the tyrosine kinase receptor muscle-specific kinase (MuSK) via its co-receptor low-density lipoprotein receptor-related protein 4 (LRP4) located in muscle cells[43], leading to the clustering of AchR in the skeletal muscle cells. MuSK also activates of rat sarcoma-related C3 botulinum toxin substrate (Rac) and Rho leading to formation of micro- and macroclusters of AChRs[44]. In ALS, the agrin-LRP4-MuSK signaling is disrupted and leads to poor formation and function of NMJs[45]. One reason for the disruption of agrin signaling is lower levels of agrin in the cerebrospinal fluid as evidenced by the prevalence of inactivating autoantibodies of the agrin and LRP4 in ALS patients[46]. Secondly, mutation in TDP-43 protein leads to downregulation of AGRN transcripts that encode agrin, and consequently lower the levels of agrin protein[47]. Agrin signaling is illustrated in Fig. 6.

Autophagy signaling occurs in motor neurons and skeletal muscle cells. The impairment in autophagy leads to neuronal death and muscle atrophy. Loss of autophagy in motor neurons accelerates denervation of NMJs in early stages of the disease and is attributed to mutation in TBK1, SQSTM1/

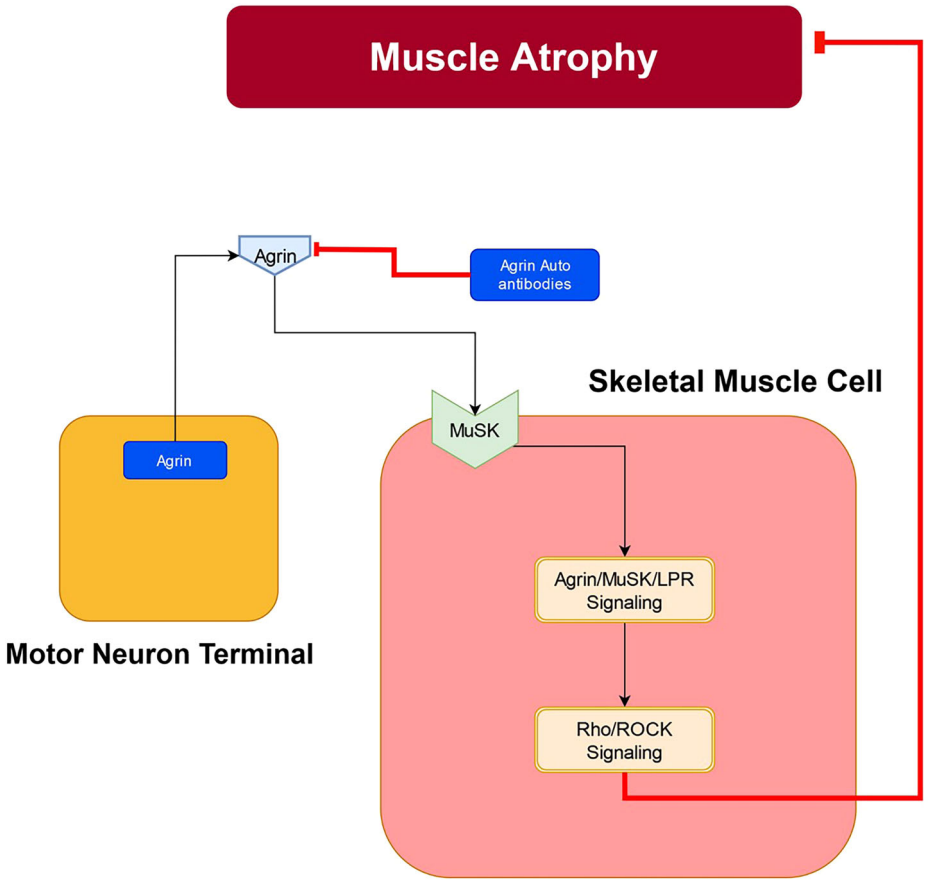


Fig. 6 | Agrin-MuSK-LPR4 signaling pathway in skeletal muscle cell. Agrin from motor neuron terminal initiates agrin-MuSK-LPR4 signaling leading to muscle atrophy via Rho-ROCK signaling in skeletal muscle cells. MuSK Muscle-specific kinase, LPR4 Low-density lipoprotein receptor-related protein 4, RhoA Rat sarcoma homolog family member A, ROCK Rho-associated protein kinase. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; small dark blue rectangle represents a protein or enzyme; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

p62, OPTN, UBQLN2, VCP, and C9orf72 genes[48,49]. The impairment of autophagy occurs via downregulation of C9orf72, which hinders the trafficking of the unc-51-like kinase 1 (ULK1) autophagy initiation complex to the phagophore by interfering with ras-related protein Rab-1A (Rab1A)-dependent processes[50].

TDP-43 is deemed essential for autophagy due to its direct interaction with autophagy related protein 4 homolog B (ATG4B) mRNA, leading to splicing[51]. Overexpression of TDP-43 revealed a suppression of autophagy caused by an increase in Bcl-2, which regulates autophagy[52]. Both over and under-expression of TDP-43 lead in corresponding increases or decreases in its functionality, and each condition on its own can inhibit the autophagy response[51]. Autophagy signaling is illustrated in Fig. 7.

Crosstalk signaling between motor neurons, skeletal muscle cells, microglial cells and astrocytes

The following are the crosstalk signaling pathways between motor neurons, skeletal muscle cells, microglial cells and astrocytes.

Glutamate metabolism in the synapse is maintained by its uptake by astrocytes via glutamate transporter EAAT2[53]. In ALS, EAAT2 expression in astrocytes is downregulated leading to synaptic accumulation of glutamate resulting in glutamate excitotoxicity and eventual neuronal death[54]. The factors affecting the downregulation of EAAT2 include the splicing errors in EAAT2, caspase 3 induced cleavage of EAAT2 C-terminus, and EAAT2 C-terminus oxidative damage that is activated by mutated SOD1[55]. EAAT2 dysregulation is implicated in both sporadic and familial ALS[56]. EAAT2 signaling is illustrated in Fig. 8.

Glutamate excitotoxicity in motor neurons is regulated by glutamate receptors present in both neurons and astrocytes cells[53]. Two receptors for glutamate, namely, α-amino-3-hydroxy- 5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-d-aspartate (NMDA) receptors are expressed by motor neurons[57]. NMDA and AMPA receptors are ligand-gated cation channels, which permit influx of Na+ and efflux of K+ across the neural synapse[57]. Reduced glutamate uptake by astrocytes due to reduced EAAT2 expression leads to accumulation of glutamate and subsequent over activation of AMPA and NMDA receptors that cause intracellular calcium overload and death of motor neurons[58]. NMDA/AMPA signaling is illustrated in Fig. 8.

Tweak exhibits abnormal expression in spinal astrocytes and skeletal muscles leading to astrocyte proliferation, IL-6 secretion in astrocytes, and motor neuron death[59,60]. Tweak–Fn14 pathway is aberrantly expressed in skeletal muscle during the course of the disease[60]. Tweak has been shown to promote ALS pathology in both the spinal cord and skeletal muscle cells. In the spinal cord, increased interferon- γ (IFN-γ) expression, a potent activator and upstream effector of Tweak, may induce up-regulation of astrocytic Tweak in late disease stages, which can subsequently participate in the maintenance of neurodegenerative and neuroinflammatory events[61,62]. In skeletal muscle cells, Tweak signaling activates canonical nuclear factor κB (NF-κB) pathway leading to upregulation of the components of ubiquitin–proteasome system (UPS) including MuRF1[63,64]. MurF1 promotes muscular atrophy via inhibition of autophagy[65]. Tweak-Fn14 signaling is shown in Fig. 9.

TGF-β plays a protective role in neurons, and its reduced signaling is linked to age-related neurodegeneration[66]. Tgfb1 mRNA increases as ALS progresses, which is attributed to reactive astrogliosis. Astrocyte-derived TGF-β accelerates ALS progression. Intracellular pathways mediating TGF-β functions in muscle cells involve alterations in suppressor of mothers against decapentaplegic (Smad) protein levels[66]. TGF-β pathway regulation at the NMJ is crucial, with TGF-β promoting synaptogenesis and influencing muscle fiber, motor neuron, and terminal Schwann cell interactions and the dysregulation in this pathway might contribute to ALS pathogenesis, impacting muscle regeneration and NMJ stability[67].

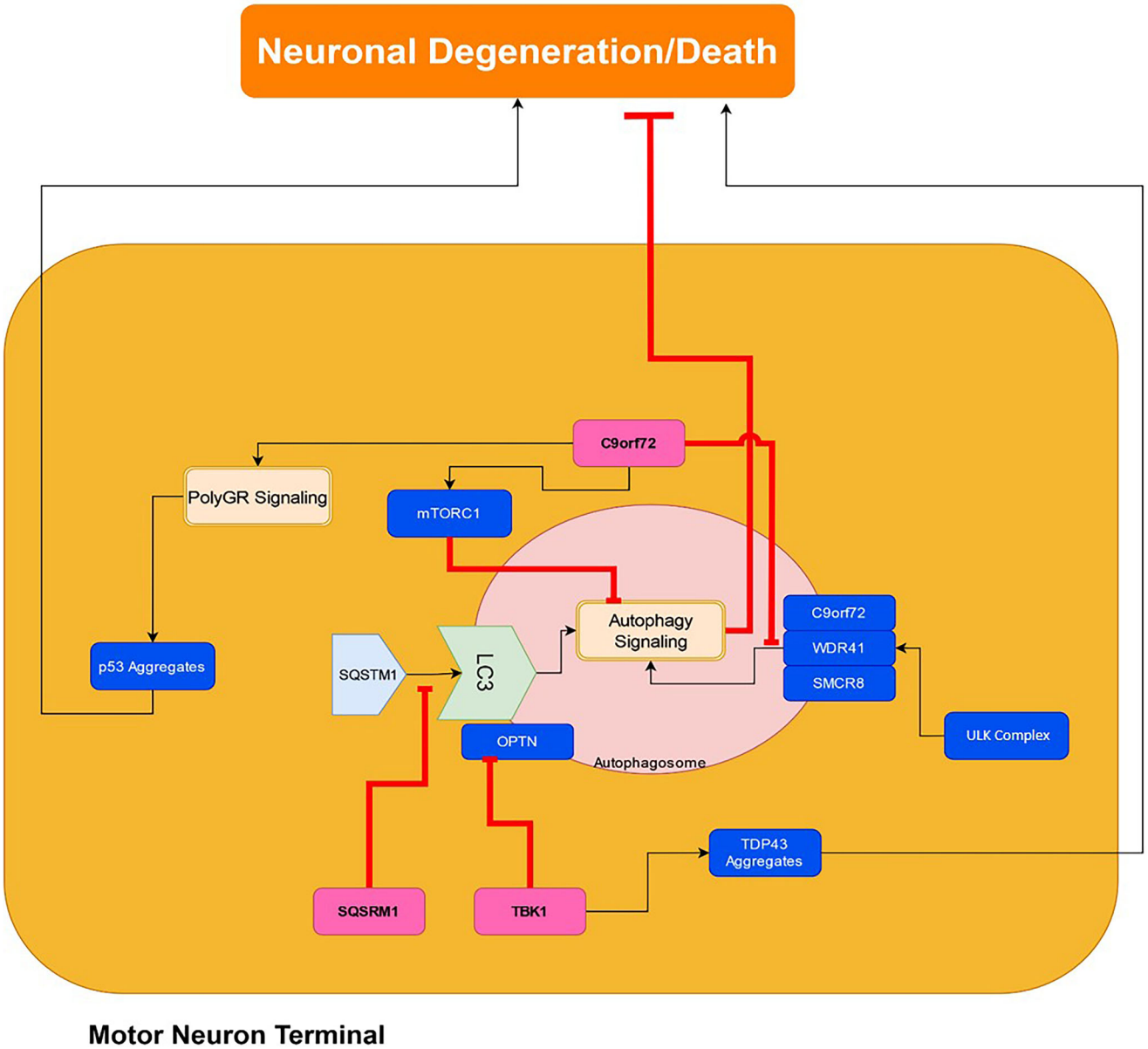


Fig. 7 | Role of autophagy in neuronal death. Mutations in genes such as SQSSRM1, C9orf72 and OPTN in ALS disrupt autophagy signaling leading to neuronal degeneration and death. mTORC1 mammalian target of rapamycin C1, OPTN optineurin, LC3 light chain 3, ULK Unc-51-like kinase, PolyGR Poly-glycine-arginine, WDR41 tryptophan-aspartic acid (W-D) repeat domain 41, SMCR8 Smith-Magenis syndrome chromosome region, candidate 8. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; small dark blue rectangle represents a protein or enzyme; small pink rectangle represents mutated protein/enzyme; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

TGF β signaling in skeletal muscle plays divergent roles depending on the pathological context and the pathway activated. In denervation states, such as with nerve injury, activation of Smads1, 5 and 8 is essential for hypertrophic signaling[68,69] whereas Smad2/3 activation promotes muscle atrophy[70]. TGF-β signaling leading to muscle atrophy is shown in Fig. 10.

Crosstalk in signaling with motor neurons, microglia, oligodendrocytes, endothelial cells and astrocytes

Communication between neurons and microglia is an essential criterion for maintaining homeostasis in the CNS. Altered crosstalk is implicated in the pathogenesis of ALS. The schematics of interactions among neuron, microglia, and astrocytes are shown in Fig. 11. Dysregulated neuron–neuron signaling and neuron–microglia crosstalk in ALS results from secretion of toxic mediators leading to reduced function and survival of motor neurons; soluble factors released by neurons and their action on microglia receptors; and, changes in direct cellular interactions.

Activated microglia and astrocytes amplify the initial damage to the motor neurons by activating activator protein 1 (AP-1) and NF-κB through production of proinflammatory cytokines and apoptosis triggering molecules such as tumor necrosis factor-α (TNF-α) and Fas ligand (FASL)[71]. ATP released during neuronal death can activate microglia and astrocytes via purinergic receptor P2X 7 (P2RX7) expressed by both glial cells leading to upregulation of major histocompatibility complex (MHC) class II proteins that are involved in presentation of antigens to T lymphocytes[72].

Pattern recognition receptors (PRRs) for pathogen associated molecular patterns (PAMPs), endogenous ligands that include Toll-like receptors (TLRs), and inflammasomes are expressed on microglia[73]. An increased expression of the nucleotide-binding domain, leucine-rich–containing

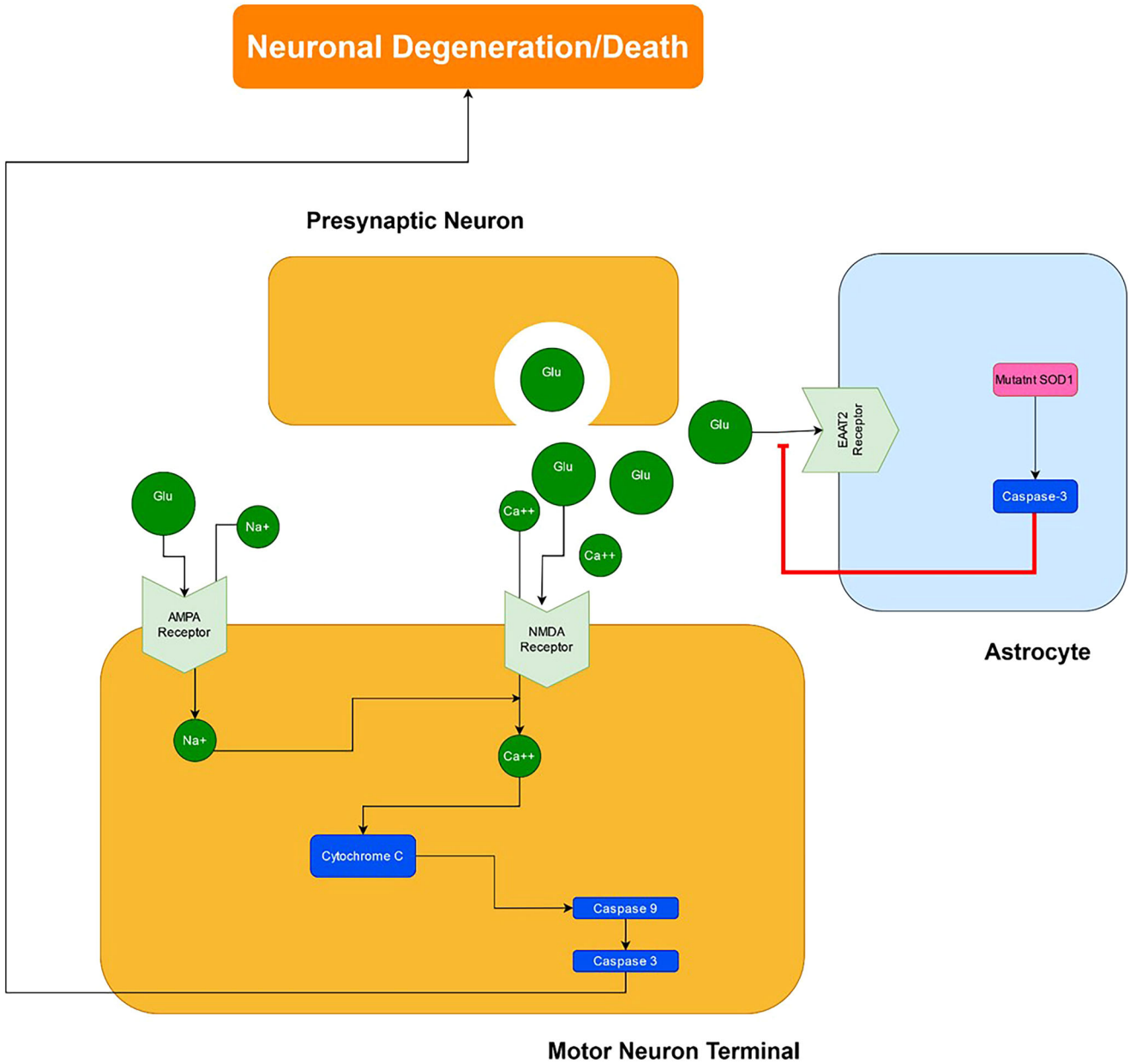


Fig. 8 | NMDA/AMPA signaling and EAAT2 signaling in neurons and astrocyte. Mutation in SOD1 dysregulates EAAT2 signaling, and consequently the dysregulation of NMDA/AMPA signaling leading to neurodegeneration due to glutamate excitotoxicity. Glu Glutamate, Na^{+} Sodium ion, Ca2 + Calcium ions. Black arrow represents signal propagation; dark green ovals represent small molecules; small dark blue rectangle represents a protein or enzyme; small pink rectangle represents mutated protein/enzyme; and, green step arrow represents a receptor.

family, pyrin domain–containing-3 (NLRP3) inflammasome is observed in both neurons and glial cells of ALS patients with cognitive impairment. The activation of the NLRP3 inflammasome was observed in $SOD1^{G93A}$ ALS-model mice astrocytes and in sporadic ALS patients[74]. This activation was accompanied by increased levels of caspase-1, IL-1β, and IL-18[71]. Inflammasome signaling is illustrated in Fig. 11.

Neuroinflammation involves the activation and proliferation of microglia and the infiltration of T cells into the brain and spinal cord[75]. In these conditions, astrocytes and microglia release proinflammatory cytokines such as IL-6[76]. In ALS, the neuroprotective phase is subsequently replaced by a cytotoxic phase, facilitated by neurotoxic microglia. The secretion of IL-6 has been found to be elevated in the cerebrospinal fluid of patients with sporadic amyotrophic lateral sclerosis[77]. IL-6 secretion from the glial cells leads to a proinflammatory state in endothelial cells leading to upregulation of proinflammatory cytokines such as TNF-α, IL-6, IL-1β, IL-8, and growth factors such as vascular endothelial growth factor (VEGF), which are found to be significantly higher in ALS patients compared to controls[78]. IL-6 signaling is illustrated in Fig. 12.

Non-neuronal cell types inside the central nervous system (CNS) play a significant role in the establishment of an extracellular environment that hinders the process of axonal regeneration. The presence of myelin debris and myelin-associated proteins resulting from the degeneration of axons has a role in impeding the process of regeneration inside the central nervous system[79]. Oligodendrocytes express myelin-associated inhibitory substances, including Nogo-A, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein[80]. These molecules engage with receptors on axons, therefore initiating the activation of RhoA/ROCK signaling pathway, which has been found to have an inhibitory effect on the

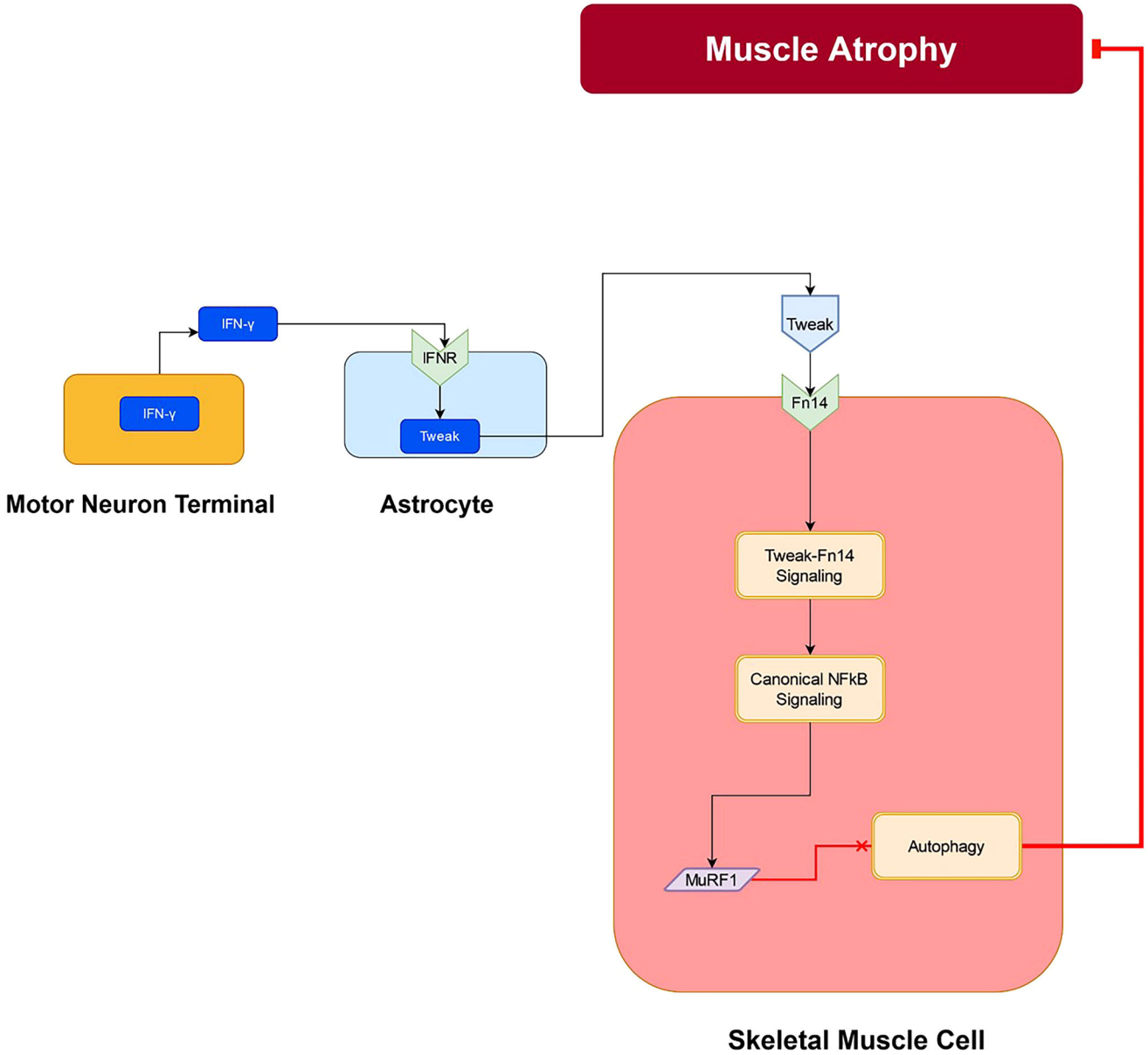


Fig. 9 | Tweak/Fn14 signaling. IFN-γ from motor neuron terminal induces Tweak production in astrocytes, which upregulates expression of MuRF1 in skeletal muscle cell. MuRF1 inhibition of autophagy in skeletal muscle cell causes muscle atrophy. IFN-γ Interferon-γ, UPS ubiquitin-proteasome system. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; small dark blue rectangle represents a protein or enzyme; purple lozenge represents mRNA; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

process of axonal outgrowth[81]. Initiation of Rho/ROCK pathway leads to inhibition of actin depolymerizing factor cofilin resulting in lower actin turnover, higher number of actin filaments, and a reduced actin turnover, resulting in inhibition of cell growth and upregulation of axonal degeneration[82]. ROCK inhibition is being studied as a potential disease-modifying therapy for ALS[83]. RhoA/ROCK signaling is illustrated in Fig. 13.

Significant findings in the recent ALS literature

Motor neurons eliminate dysfunctional mitochondria by recycling their components when they are irreparably damaged by a process known as mitophagy, a specific form of autophagy[84]. The loss of mitophagy is increasingly recognized as a characteristic feature of neurodegenerative diseases[84]. In ALS patients, mutations in mitophagy regulatory genes such as optineurin and p62/sequestrosome-1 lead to loss of mitophagy[85]. Levels of mitophagy regulatory protein translocator protein (TSPO) are higher in ALS condition, which leads to reduction in autophagy marker autophagy related gene 12 (Atg12) levels and subsequent suppression of mitophagy[85]. The increase in expression of TPSO has been attributed to over activation of extracellular signal-regulated kinase (ERK) 1/2 pathway, accumulation of mutant SOD protein aggregates, and reduced Sirt3 levels[85,86]. Dysfunction in mitophagy leads to cell death[84]. Mitophagy signaling in motor neurons is represented in Fig. 14.

MicroRNAs (miRNAs) are small RNAs that are critical in regulation of post-translational gene expression[87]. In ALS, miRNAs are being utilized as markers of diagnosis and monitoring of disease progression[88]. A list of miRNAs associated with ALS NMJ microenvironment and their targets is shown in Supplementary Table 2. Additionally, use of miRNA as potential therapeutic target is currently being investigated[89,90]. Astrocytes derived from both sALS and fALS fibroblasts exhibited abnormal expression and release of miRNA146a[91]. The expression of miRNA146a may vary in

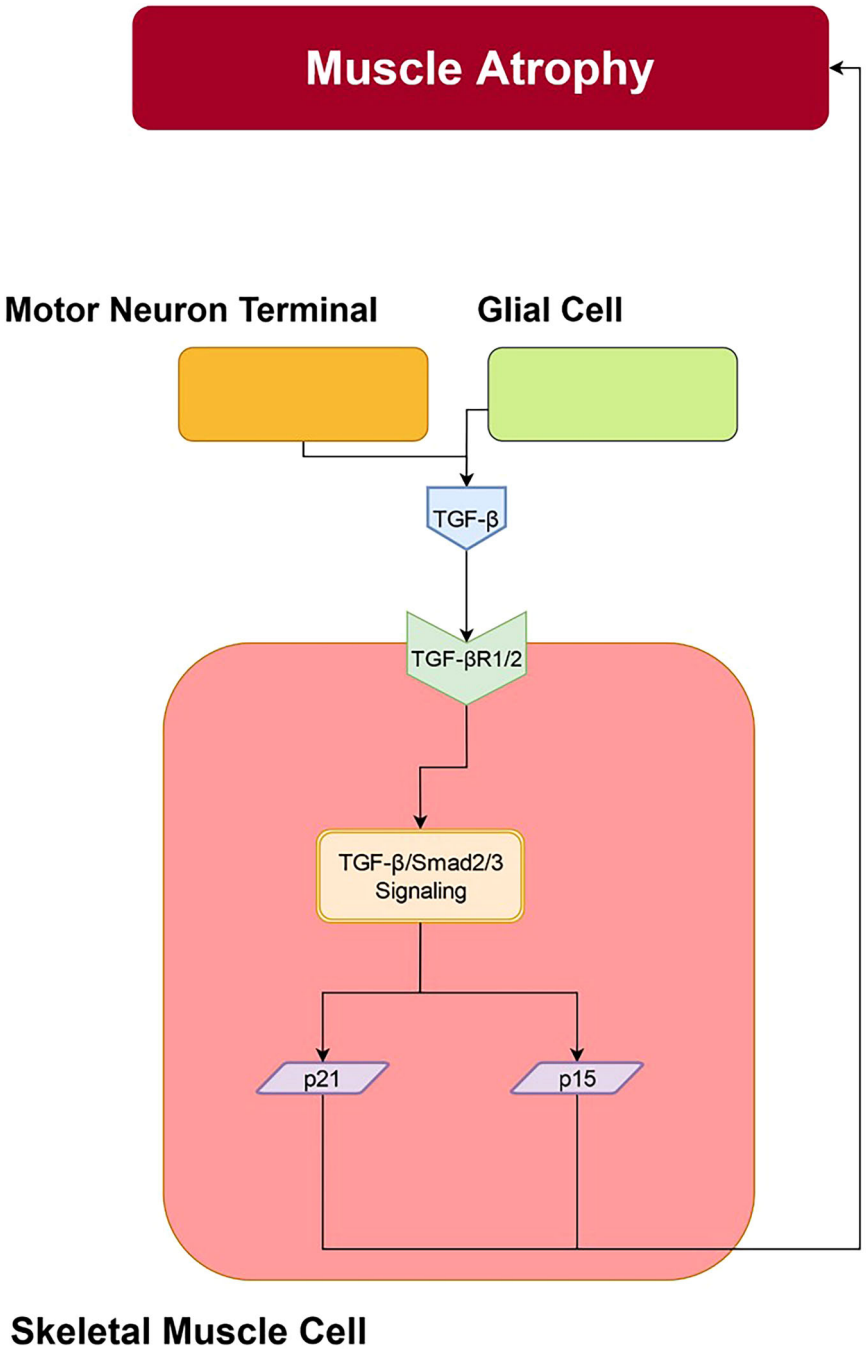


Fig. 10 | TGF-β signaling. Activation of Smad2/3 by TGF-β promotes muscle atrophy via expression of p21 and p23. TGF-βR - TGF-β receptor; Smad Suppressor of mothers against decapentaplegic, p21 Protein p21, p15 Protein p15. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; purple lozenge represents mRNA; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

patients, either being reduced or enhanced. Replenishing miRNA146a levels in depleted astrocytes affected by ALS reversed their reactive and inflammatory phenotype[92]. miRNA146a regulates the expression of several genes, including TNFR-associated factor 6 and IL-1R-associated kinase, which are known to activate the NF-κB inflammatory pathway[93], a key contributor of neuroinflammation. miR-494-3p is an astrocyte-derived microRNA that is released in exosomes and its expression is decreased in cells derived from patients with C9orf72 hexanucleotide repeat expansion (HRE) and in the spinal cord of individuals with sporadic amyotrophic lateral sclerosis (sALS)[94]. Restoring miR-494-3p levels resulted in the expression of Sema3A, a crucial gene for maintaining motor neuron axons[94].

Discussion

This study hypothesized that a molecular systems architecture of the NMJ microenvironment may provide a systems-level understanding of ALS pathogenesis. The results from this effort provide: a multi-layered visual map of biomolecular interactions in the NMJ microenvironment, an understanding of the complex crosstalk in the NMJ microenvironment, potential targets for drug development, and a framework to develop computational models providing quantitative predictions.

One of the results from this study is a multi-layered molecular systems architecture to visualize molecular interactions within and across the cells of the NMJ microenvironment. These molecular interactions – as shown in Fig. 3 – occur across the motor neurons, the microglia, astrocytes, oligodendrocytes, terminal Schwann cells or skeletal muscle cells. This visual map can be used to identify how perturbations to a molecular species e.g., protein, receptor, promoter, gene, mRNA, etc., in one cell may impact the biological functions in a neighboring microenvironmental cell, culminating in inhibiting or activating a biological process such as neuronal degeneration/death, muscle atrophy, and neuroinflammation leading to ALS

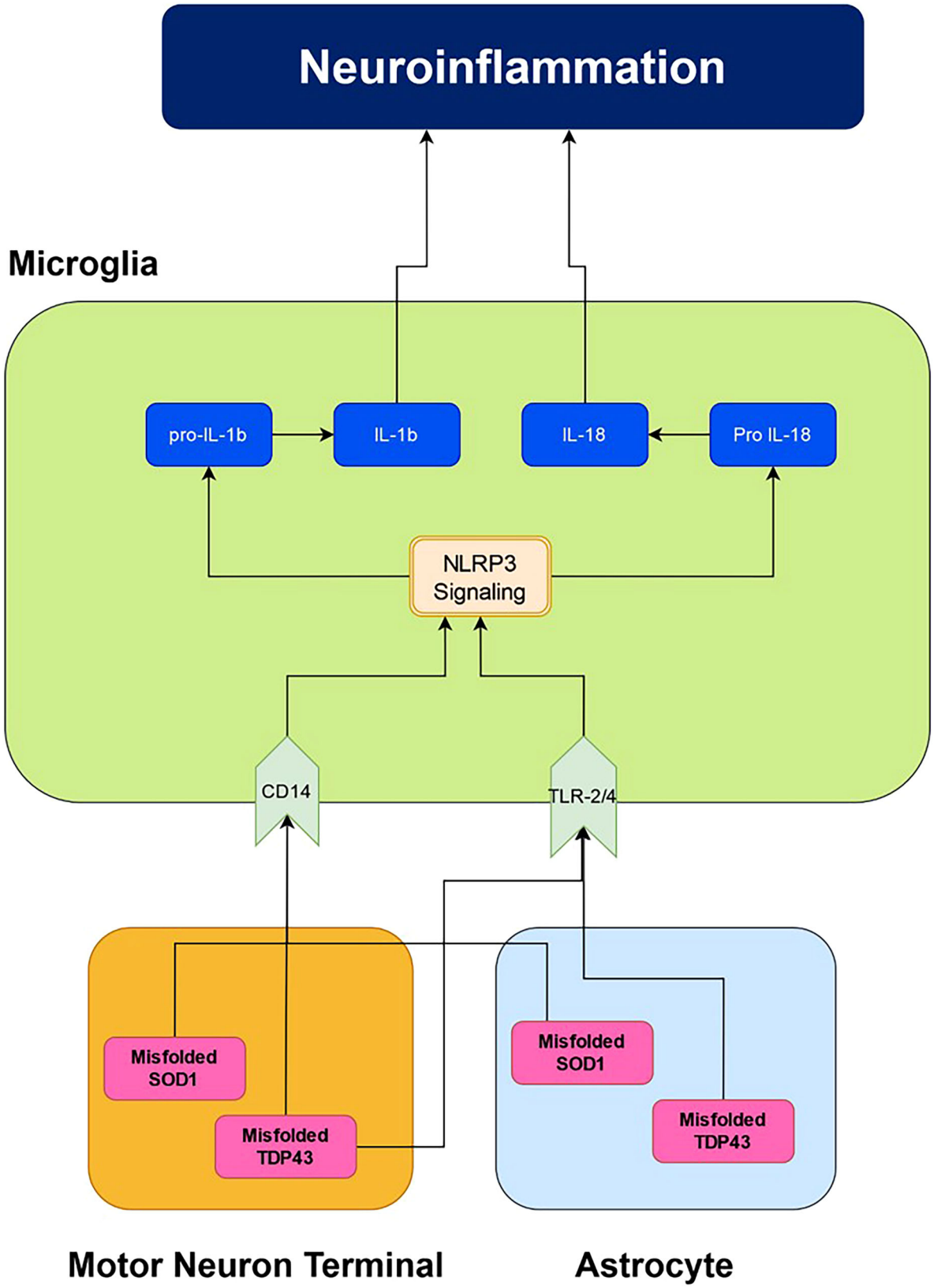


Fig. 11 | Interactions between astrocytes, microglia and neurons leading to neuroinflammation. Misfolded proteins from motor neurons and astrocyte activate the CD14 and TLR receptors on microglia to initiate NLRP3 inflammasome signaling leading to neuronal inflammation. NLRP3 nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3, TLR Toll-like receptor; CD14 cluster of differentiation 14, IL-1b Interleukin 1β, IL-18 Interleukin 18. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; small dark blue rectangle represents a protein or enzyme; small pink rectangle represents mutated protein/enzyme; and, green step arrow represents a receptor.

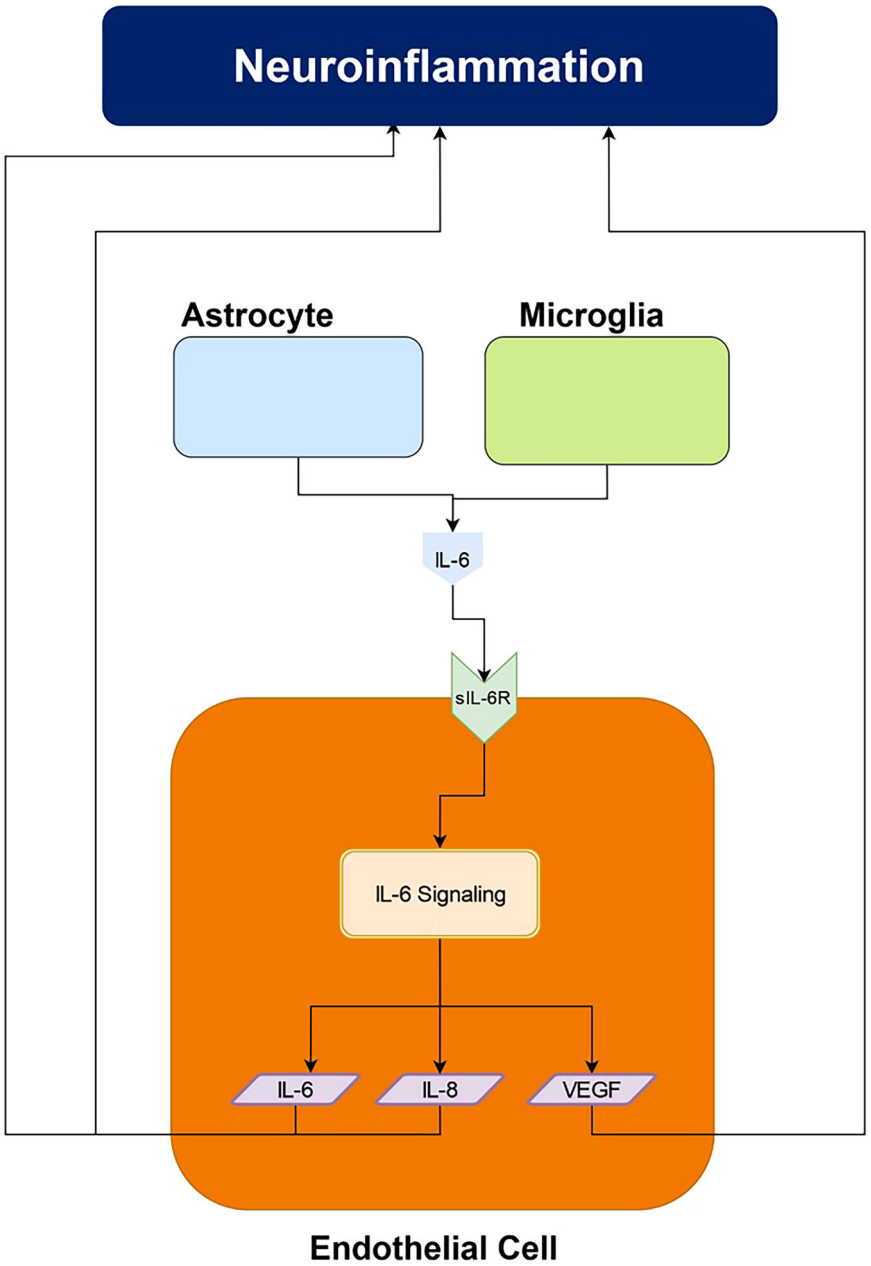


Fig. 12 | IL-6 signaling. IL-6 secretion from the glial cells promotes upregulation of proinflammatory cytokines such as TNF-α, IL-6, IL-8, and growth factors such as VEGF and in endothelial cells, leading to neuroinflammation. IL-8 Interleukin 8, TNF-α Tumor necrosis factor-α, VEGF Vascular endothelial growth factor. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; purple lozenge represents mRNA; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

pathogenesis. An effort is currently underway to make this molecular systems architecture web-enabled so the ALS research community may navigate and explore the multiple layers of the molecular systems architecture as well as interact and provide feedback to update existing versions to reflect advances from ongoing research.

In addition to a visual multi-layered map of biomolecular interactions provided by the molecular systems architecture, the architecture has also revealed the crosstalk across the seven different cells in the NMJ microenvironment. Across motor neurons and skeletal muscle cells, the architecture identified the following three crosstalk signaling pathways: ER stress signaling, agrin signaling, and autophagy signaling. Among motor neurons, skeletal muscle cells, microglial cells, and astrocytes, the architecture identified the following four crosstalk signaling pathways: EAAT2 signaling, NMDA and AMPA signaling, Tweek/Fn14 signaling, and TGF-β signaling. Among motor neurons, microglia, oligodendrocytes, endothelial cells and astrocytes, the architecture identified the following three crosstalk signaling pathways: inflammasome signaling, IL-6 signaling, and RhoA/ROCK signaling. These insights provide an integrative whole systems understanding of ALS pathogenesis.

The molecular systems architecture of ALS herein has provided potential therapeutic targets for drug development. This architecture reveals several targets across different cell types in the NMJ microenvironment that can be inhibited or activated to affect the biological processes of neuronal degeneration/death, muscle atrophy, and neuroinflammation. These potential targets were itemized in Table 2. The targets were categorized according to one of the three biological processes identified in the molecular systems architecture in Fig. 2. A total of sixteen molecular targets were identified within the molecular pathways that are involved in neuronal degeneration, muscle atrophy, and neuroinflammation across NMJ microenvironmental cells. Among these sixteen molecular targets, seven were identified in motor neurons and two in astrocytes that are implicated in the molecular pathways involved in neuronal degeneration/death; five targets were identified in skeletal muscle cells that are involved in the molecular pathways of muscle atrophy; and, finally, three targets were identified in

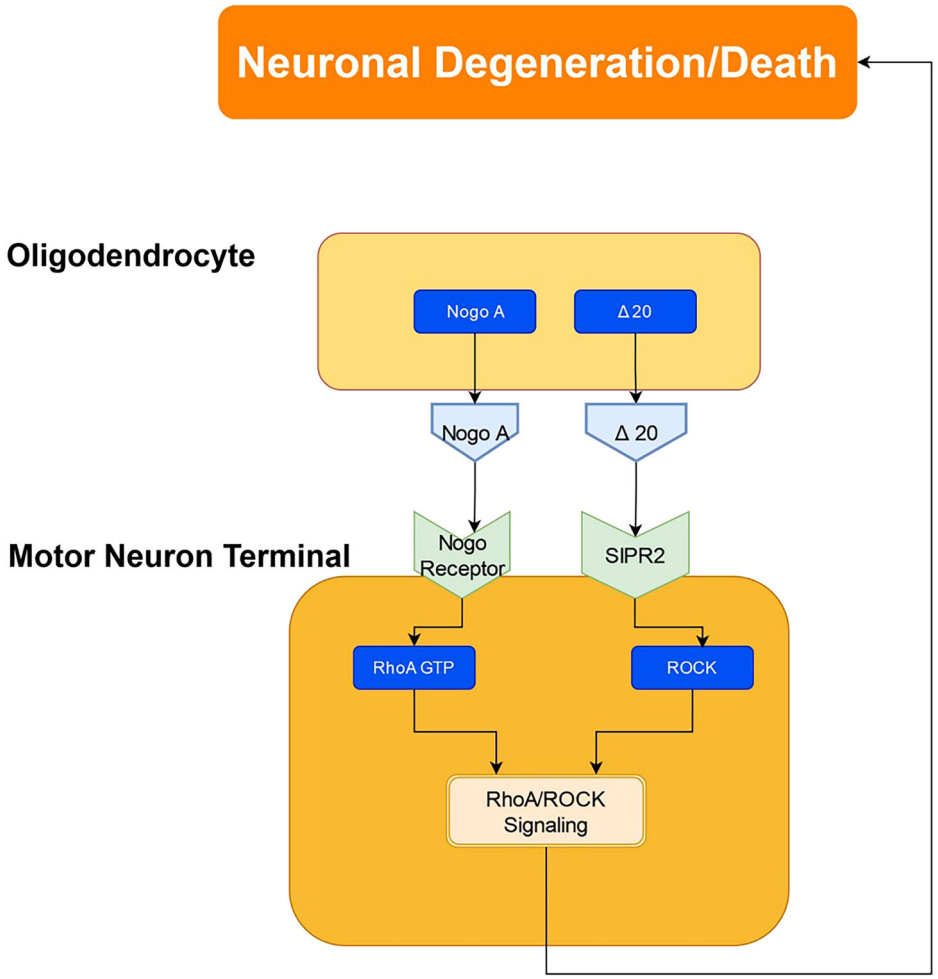


Fig. 13 | RhoA/ROCK signaling. Myelin-associated inhibitory substances from oligodendrocytes initiate Rho/ROCK signaling in motor neurons, leading to inhibition of cell growth and axonal degeneration, thereby destabilizing the NMJ. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; small dark blue rectangle represents a protein or enzyme; light blue pentagram represents the ligand; and, green step arrow represents a receptor.

microglia that are involved in the molecular pathways of neuroinflammation.

Conventional drug development aims to identify a single compound that affects a particular target to inhibit or delay ALS progression. The molecular systems architecture of the NMJ microenvironment in ALS may enable such development to proceed with a more conscious systems-level understanding. For example, the molecular systems architecture may be used to identify how activating or inhibiting more than one target using multi-combination therapies can affect multiple biological processes implicated in ALS pathogenesis. In addition, the visual map could provide insights on how targeting one or more mechanisms in the NMJ microenvironment affects the biological process in either a positive or negative therapeutic outcome. Such insights may also include understanding how a therapeutic that has beneficial effect on a target in a particular cell, may interact with another target in a different cell of NMJ microenvironment to lead to adverse effects on treatment outcome.

Mechanistic computational modeling is emerging as a valuable preclinical therapeutic discovery tool. The molecular systems architecture may provide a framework for developing in silico models of molecular pathways in the NMJ microenvironment. Such in silico models of NMJ microenvironment may be used to answer research questions, such as, of the targets identified by the molecular systems architecture in Table 2, which ones will prove more efficacious in reducing neuroinflammation, muscular atrophy, or neuronal degeneration. In addition, such in models may be used to provide quantitative predictions for applications such as efficacy and safety of single and multi-combination therapeutics, and determination of optimal dosages. In previous work, a molecular systems architecture of pancreatic cancer was successfully used to develop computational models that predicted dosages of a two-drug combination that was allowed by the U.S. Food and Drug Administration (FDA)[95]. Additionally, in silico models based on a molecular systems architecture of osteoarthritis were used to derive an optimal combination of two bioflavonoids targeting molecular pathways of pain and inflammation[96].

Methods

The scientific literature is searched to identify journal papers that contain research on ALS, molecular pathways of ALS, cells in the ALS neuromuscular microenvironment, and the molecular pathways involved in the cellular crosstalk in the ALS neuromuscular microenvironment. The CytoSolve® systems biology tool used in this study enables the systematic bioinformatics literature review as well as scalable computational modeling of molecular pathways[9,97–100]. CytoSolve has been applied to diverse areas in systems biology such as osteoarthritis[101], neurovascular diseases[5], acute myeloid leukemia[6], periodontitis[7], to name a few. The CytoSolve® Operating Guide, used in the methodology herein, has been previously published[102–104].

Literature review methodology

The development of a systems architecture includes exploring the public repository of scientific papers. CytoSolve[97], a systems biology tool, is used to facilitate the systematic bioinformatics literature review process as well as scalable computational modeling of pathways derived from such review. CytoSolve's capabilities for systematic bioinformatics literature review allow for curation, archival, screening, and distributed collaborative review of the

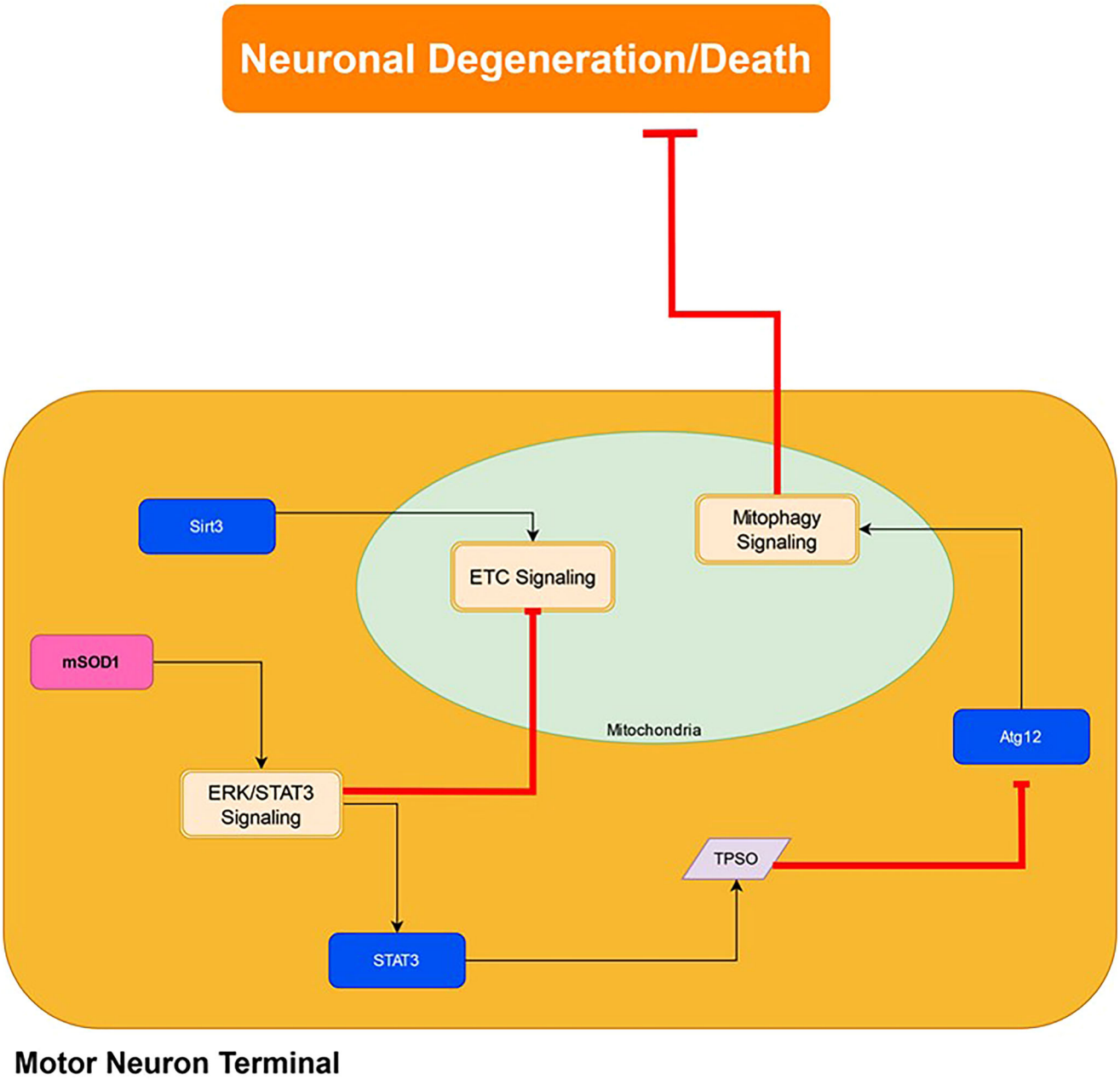


Fig. 14 | Mechanisms of mitophagy in neurons. Mutant SOD aggregates inhibition ETC signaling and autophagy via ERK/STAT3 signaling and Atg12 suppression, respectively. Autophagy suppression leads to neuronal cell death. ETC Electron transport chain, ERK Extracellular signal-regulated kinase, STAT3 signal transducer and activator of transcription,- Atg12 Autophagy related gene 12, TPSO Translocator protein. Double-sided orange rectangle represents a molecular pathway within a cell; black arrow represents signal propagation; red flat arrow represents inhibition of signal propagation; small dark blue rectangle represents a protein or enzyme; small pink rectangle represents mutated protein/enzyme; and, purple lozenge represents mRNA.

selected relevant literature. Specifically, the algorithm was used to distill the relevant set of literature included following steps:

1. Creating a list of Medical Subject Headings (MeSH) keywords to optimize recall and precision of peer-reviewed articles
2. Searching and retrieving the relevant peer-reviewed articles published between January 1980 to July 2023 from PubMed, Medline, and Google Scholar. These articles are stored as an "Initial Set" repository
3. Screening the titles and abstracts of articles in the Initial Set repository to identify the most relevant articles based on our inclusion criteria. This subset of articles is then stored as the "Final Set" repository
4. Performing a full-length review of peer-reviewed articles from the Final Set repository

Inclusion and exclusion criteria

The full text of the articles, not only the abstracts, are reviewed completely by the authors. An article is deemed relevant only if the body of the article contains the keywords in Supplementary Table 3 with specific relation to ALS pathogenesis. In the screening process, abstracts and unpublished literature were not sought, as they have not been peer reviewed adequately to authenticate their results. A list of Medical Subject Headings (MeSH) keywords to optimize recall and precision of peer-reviewed articles is provided in Supplementary Table 3. The CytoSolve systematic bioinformatics literature review process inclusion criteria and categorization are represented in Fig. 15 as per the PRISMA guidelines[105]. The PRISMA checklist is included in Supplementary Table 4 and Supplementary Table 5.

Table 2 | Summary of potential therapeutic molecular targets

Molecular Target	Cell Type	Biological Process	Reference
ATG7	Motor neurons	Neuronal Degeneration/Death	58,120
ROCK	Motor neurons	Neuronal Degeneration/Death	82
Manganese superoxide dismutase	Skeletal muscle	Muscle Atrophy	116
Dynactin1a	Motor neurons	Neuronal Degeneration/Death	121
Mitochondrial uncoupler protein (UCP1)	Skeletal muscle	Muscle Atrophy	122
CTGF/CCN2	Skeletal muscle, astrocytes and motor neurons	Muscle Atrophy, Neuronal Degeneration/Death	123
SARM-1	Motor neurons	Neuronal Degeneration/Death	119
Oxidation resistance 1 (Oxr1) protein	Motor neurons	Neuronal Degeneration/Death	124
PDK4	Skeletal muscles	Muscle Atrophy	125
IL-1α, TNF and C1q	Microglia	Neuroinflammation	126
HDAC4	Skeletal muscle cells	Muscle Atrophy	90
NOX2	Motor neurons	Neuronal Degeneration/Death	125
KIF5A	Astrocytes	Neuronal Degeneration/Death	127
5-Hydroxytryptamine (5-HT)	Motor neurons	Neuronal Degeneration/Death	128

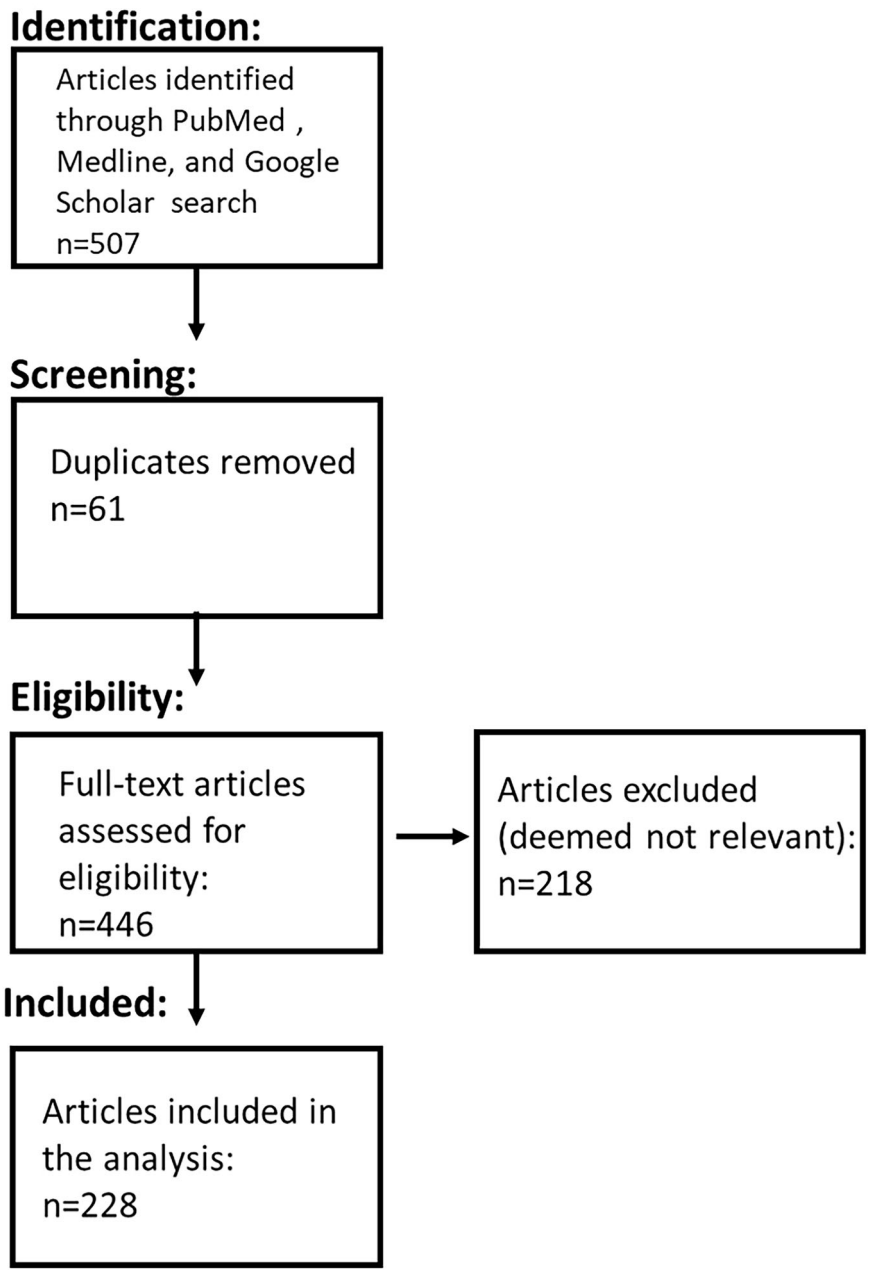


Fig. 15 | PRISMA flow diagram. In the process above, 507 articles are identified; 61 duplicates were removed; 446 articles were eligible for review from which 218 were removed as they were deemed not relevant; and, 228 articles were included in the analysis.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Author contributions

Conceptualization by V.A.S., P.D., and R.D.K.; systematic literature review, visualization, and by P.D.; Writing (first draft) by P.D.; Writing (review and editing) by P.D., V.A.S. and R.D.K.; Resources, supervision, project administration, by V.A.S., P.D., and R.D.K.

Competing interests

V.A.S. Ayyadurai, and P. Deonikar are employees of CytoSolve, Inc. R.D. Kamm is an employee of Massachusetts Institute of Technology.

Additional information

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