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The Complex Molecular Biology of Amyotrophic Lateral Sclerosis (ALS)

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder that causes selective death of motor neurons followed by paralysis and death. A subset of ALS cases is caused by mutations in the gene for Cu, Zn superoxide dismutase (SOD1), which impart a toxic gain of function to this antioxidant enzyme. This neurotoxic property is widely believed to stem from an increased propensity to misfold and aggregate caused by decreased stability of the native homodimer or a tendency to lose stabilizing posttranslational modifications. Study of the molecular mechanisms of SOD1-related ALS has revealed a complex array of interconnected pathological processes, including glutamate excitotoxicity, dysregulation of neurotrophic factors and axon guidance proteins, axonal transport defects, mitochondrial dysfunction, deficient protein quality control, and aberrant RNA processing. Many of these pathologies are directly exacerbated by misfolded and aggregated SOD1 and/or cytosolic calcium overload, suggesting the primacy of these events in disease etiology and their potential as targets for therapeutic intervention.

I. ALS Is a Deadly Neurodegenerative Disorder

Amyotrophic lateral sclerosis (ALS) was first described by the noted French neurologist Jean-Martin Charcot in 1869, who connected the progressive paralytic syndrome with lesions in both white and gray matter of the central nervous system (CNS).[1] Over 140 years later, ALS is the most common adult-onset motor neuron disorder, affecting approximately 1–2 per 100,000 people worldwide. Considering the short course of disease progression (death/tracheotomy typically within 2–5 years of diagnosis), 1 in every 800 individuals is expected to face ALS in his/her lifetime.[2–4]

As described by Charcot, ALS involves degeneration of the upper motor neurons (UMN) of the motor cortex and of the lower motor neurons (LMN), which extend through the brainstem and spinal cord to innervate skeletal muscle. Though the upper and lower motor systems are known to be interconnected, controlling voluntary muscle movement in concert, the primary site of dysfunction in ALS has long been a source of debate.[5–7] Questions of UMN/LMN primacy aside, ALS is clearly specific for motor neurons and largely spares cognitive ability, sensation, and autonomic nervous functions. Muscles controlling eye movement and the pelvic floor are the only skeletal musculature left unaffected. However, in a minority of cases (5–10%), patients also develop frontotemporal lobar dementia (FTLD). It has been suggested that a greater percentage of patients experience some cognitive change (such as loss in executive function) without crossing the threshold required for a diagnosis of dementia.[8]

Clinical presentation varies but most commonly consists of weakness, fasciculations (twitching muscles), and/or hyperreflexivity of facial muscles (bulbar onset) or limbs (spinal onset). Interestingly, initial symptoms usually appear at a focal site and later spread along contiguous anatomic paths.[9] Diagnosis is achieved by a combination of clinical examination and electromyography (EMG), in which positive, sharp waves and fibrillation potentials provide evidence for active denervation. The El Escorial criteria were developed in 1990 and are still utilized to diagnose and classify ALS cases as "possible," "probable," or "definite"[10] (Fig. 1). Guidelines on implementation of the El Escorial criteria have been revised to place greater emphasis on electrophysiological abnormalities, which can be detected earlier and thus facilitate timely diagnosis.[11]

II. Etiology of ALS

The majority of ALS cases (≈82%) are sporadic (SALS), having no apparent heritability.[9] Up to 5% of SALS cases are caused by mutations in the 43-kDa *trans*-activating response region DNA-binding protein (TDP-43). TDP-43 mutations have also been linked to ≈3% of inherited, or "familial" ALS (FALS).[12] The most commonly occurring mutations in patients with FALS are found in the gene for Cu, Zn superoxide dismutase (SOD1) and account for approximately 20% of all FALS.[13,14] Most of these mutations are missense mutations that cause autosomal dominant ALS, except the D90A polymorphism, which can also behave as a recessive mutation.[15] FALS-causative mutations have also been found in genetic loci corresponding to alsin, a guanine exchange factor for Rac1 that plays a role in cytoskeletal dynamics[16,17]; senataxin, a DNA/RNA helicase that may be involved in RNA processing[18,19]; vesicle-associated membrane protein-associated protein B (VAPB), which facilitates intracellular vesicular trafficking[20]; and angiogenin (ANG)[21–23] (Table I). Some polymorphisms found in patients with ALS do not segregate completely with disease and may represent genetic risk factors rather than causative mutations. Mutations in the neurofilament-heavy subunit,[24,25] vascular endothelial growth factor (VEGF),[26] and ciliary neurotrophic factor (CNTF)[27,28] fall under this category. All genetic loci that have been reported as putative modifiers of ALS susceptibility are listed in the ALS Online Genetics Database (http://alsod.iop.kcl.ac.uk).

There is evidence to suggest that specific environmental factors play a prominent role in the etiology of some ALS cases. Geographically limited populations with dramatically increased ALS incidence, such as inhabitants of the Kii peninsula in Japan,[29] the Chamorro people of Guam, Gulf War veterans,[30,31] and Italian soccer players,[32] certainly lead one to suspect the environment as a potential modifier of disease susceptibility. There also have been reports of ALS in individuals with intense exposure to particular stressors, such as harsh chemicals and heavy metals,[33,34] viral infection,[35] electrical shock,[36] and traumatic nerve injury.[37] Most of these reports, however, involve a very small number of cases and do not permit rigorous evaluation of these stressors as potential risk factors for ALS.

The most convincing instance of a causal link between ALS and toxin exposure is the case of the Chamorro population. A cycad indigenous to Guam produces the neurotoxin β-methylamino-L-alanine (BMAA) in its seeds, which are eaten by flying foxes as well as ground into flour by the Chamorro. While the dosage of BMAA resulting from a reasonable human consumption of cycad flour is far below the threshold necessary to provoke neurodegeneration in primates,[38] this potent neurotoxin is enriched 100-fold in the tissues of the flying fox, a delicacy to the Chamorro.[39,40] Furthermore, BMAA is found in the brain tissue from Chamorros who succumb to ALS, but not those who die of other causes, and the prevalence of ALS among this population dropped after overhunting thinned the flying fox population.[39] While providing a convincing causal link between BMAA exposure and ALS, it is tempting to dismiss the case of the Chamorro as inapplicable to disease risk in the

general population. However, BMAA-producing cyanobacteria are present in many ecosystems, and a recent study of Baltic Sea marine life revealed that BMAA is concentrated in the tissues of organisms at higher trophic levels, such as fish and mollusks, that are consumed by humans.[41] BMAA also was found in cyanobacteria-containing sand from Qatar,[42] raising the possibility that Gulf veterans may have been exposed to this toxin through inhalation. Furthermore, the incidence of ALS diagnosis is elevated 10–25–fold among residents of Enfield, New Hampshire, a town bordering Lake Mascoma, which is subject to frequent “blooms” of cyanobacteria.[43] While no conclusive statements can be made from these few examples, the worldwide prevalence of cyanobacteria seems a compelling reason to investigate ALS risk associated with BMAA exposure.

III. SOD1-Related Pathology as a General Model for ALS

The discovery of SOD1's role in FALS[14] offered the first insight into the molecular mechanisms of ALS, and the study of SOD1-mediated pathology has contributed much to our current understanding of the disease. The majority of *in vivo* work has utilized transgenic mice expressing FALS mutants of human SOD1, which develop a progressive motor neuron syndrome reminiscent of the human ALS phenotype (reviewed in Ref. 44). The sporadic disease differs little clinically from SOD1-related FALS, leading to the widespread supposition that all cases of ALS share some common mechanism(s) of pathology.[2,45,46] In reviewing the proposed molecular bases of ALS, we focus on the contribution of SOD1, a well-studied cause of ALS that may exhibit pathogenic mechanisms common to other forms of the disease.

IV. Misfolding and Aggregation Is the Most Likely Source of SOD1 Toxicity

SOD1 is a ubiquitous cytosolic enzyme whose primary function is the dismutation of the superoxide radical $\left(O_2^{\cdot-}\right)$ to a less oxidizing species (H_2O_2) via a bound Cu^{2+} ion. Although this enzyme plays an important role as a cellular antioxidant, the ability of SOD1 mutants to selectively kill motor neurons is not linked to a loss of dismutase function. Not only do many FALS mutants retain enzymatic activity at or near wild-type levels,[47–49] but SOD1 null mice do not exhibit neurodegeneration.[50] Furthermore, the toxicity of SOD1 mutants cannot be reversed by coexpression of wild-type SOD1.[51] This evidence has led to widespread acceptance of the hypothesis that SOD1 mutants acquire a novel toxic property independent of their enzymatic function.

Despite over 15 years of research, the mode(s) by which SOD1 mutants selectively kill motor neurons have not been clearly delineated. However, a large body of evidence implicates a common propensity to misfold and aggregate as the primary toxic gain of function. Destabilization of the native fold is an attractive hypothesis for SOD1 mutant pathogenicity, offering a plausible explanation for the common disease outcome of over 140 mutants spanning the sequence and structure. Early *in silico* studies by our laboratory predicted that a majority of SOD1 mutations would destabilize the native fold or quaternary structure,[52] a trend that since has been verified experimentally.[53–56] Especially severe destabilization caused by certain mutations could account for their inherently higher aggressiveness (short disease duration).[57,58] Indeed, several recent analyses of *in vitro* SOD1 mutant behavior and FALS patient survival showed that protein instability and increased aggregation rate correlated with decreased survival time[59,60] (Fig. 2). Furthermore, the presence of SOD1-immunoreactive proteinaceous aggregates in SALS patient motor neurons[62–65] suggests that aberrant oligomerization of SOD1 could be a common feature of ALS, regardless of genotype. It thus appears that ALS is a protein conformational disorder, akin to other neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.[2]

Though a primary role for SOD1 aggregation in FALS seems likely, deconstruction of the molecular determinants and mechanisms of this process is incomplete. SOD1 is an extremely stable enzyme in its fully mature, homodimeric form, remaining active in the presence of 6M guanidinium chloride or 8M urea.[66,67] SOD1 owes its extraordinary stability largely to the coordination of $Zn^{2þ}$, which constrains the relatively unstructured electrostatic and zinc-binding loops, “tethering” them together and protecting the protein core, an eight-stranded Greek key β-barrel[53,68,69] (Fig. 3). The catalytic copper ion and an intrasubunit disulfide bridge between Cys-57 and Cys-146 appear to contribute relatively little to monomer thermodynamic stability, but the latter modification constrains loop mobility and facilitates dimer formation.[66,68,74] Metal-bound, disulfide-oxidized SOD1 forms an exceptionally stable homodimer, with low nanomolar binding affinity.[75,76] These maturation events are mutually interdependent—metal binding promotes disulfide bond formation, disulfide bond formation and metal binding promote dimerization, and dimeric SOD1 is more resistant to disulfide reduction/metal loss.[68,75,77]

In vitro studies show that dimer dissociation is a necessary initiating step in SOD1 aggregation.[76,78] The resultant monomeric SOD1 is more susceptible to the loss of the stabilizing zinc ion and disulfide bridge,[79,80] leading to freer loop movement[81] and exposure of β-barrel edge strands.[68,82] Dynamical studies of wild-type and FALS mutant SOD1 revealed a transient “excited state” whose population is enhanced by mutations and zinc loss, but unaffected by disulfide status.[83] Increased surface hydrophobicity of metal-free, disulfide-reduced mutant SOD1 was shown directly by Tiwari *et al.* using 1-anilinonaphthalene-8-sulfonic acid (ANS), a fluorescent dye that binds to hydrophobic surfaces.[84] Munch *et al.* obtained similar results using a different hydrophobic dye, Sypro Orange, and found that increased exposure of hydrophobic regions precedes aggregation.[85] A general model of SOD1 aggregation in ALS has emerged in which dimer dissociation and subsequent metal loss (and/or disulfide reduction) induce structural distortions that favor assembly into non-native oligomers (oligomers other than the native homodimer) (Fig. 4). FALS mutations promote aggregation by increasing the tendency of SOD1 to lose its stabilizing posttranslational modifications and/or by decreasing the intrinsic stability of the apo-monomer.[52,54–56,68,86–88] Substantial gaps remain in our understanding of the relationship between SOD1 aggregation and ALS pathology. These pertain to aggregate structure, mechanism of formation, and toxicity.

A. SOD1 Aggregate Structure

No high-resolution structural information is available for misfolded monomeric SOD1 or nonnative oligomers. The transient nature of many structurally-perturbed SOD1 species makes their isolation for study impractical. However, misfolded dimeric or monomeric SOD1 can be detected using an antibody specific for residues 145–151, which are normally buried within the native dimer interface.[89] SOD1 monomers with a more substantially disrupted fold can be tracked using an antibody recognizing the natively buried residues of β strand 4 (residues 42–48).[90] Chromatographic methods have also been utilized to isolate misfolded SOD1 using their affinity to hydrophobic resins.[91] Continued study using these and similar methods will be useful in tracking the spatial and temporal distribution of misfolded SOD1 in cell culture, transgenic mouse models, and patients with ALS, providing insight into the molecular determinants and cellular consequences of SOD1 destabilization.

Electron microscopic, immunohistological, and biochemical studies have shed some light on the structural properties of SOD1 aggregates. Both insoluble, detergent-resistant aggregates and soluble oligomers have been noted in cell culture, transgenic mice, and *in vitro*.[63,64,92–94] These species contain metal-free SOD1 that is full length and usually lacks the native disulfide bridge.[95] Aggregates formed *in vitro* under near-physiological conditions are often fibrillar and bind thioflavin T (ThT^{+}, suggestive of amyloid

character),[86,96–98] while *in vivo* aggregates sometime appear amorphous or pore-shaped and do not always bind amyloid-sensitive dyes.[90,93,99–101] Soluble misfolded SOD1 populates a wide range of oligomeric states and also accumulates as non-native monomers, dimers, or trimers.[62,91,96] The instability of some soluble oligomers may preclude the use of static structural techniques, such as X-ray crystallography, to determine structural details, but solution-state methods such as nuclear magnetic resonance (NMR) or limited proteolysis, especially coupled with computational structural modeling, may yield insights into their conformations.

B. Mechanism of SOD1 Aggregation

The likelihood that misfolded SOD1 samples a multitude of conformational states also complicates detailed mechanistic study of oligomer formation. However, it is clear that posttranslational modifications of the SOD1 polypeptide modulate oligomer formation to some extent. As discussed above, the native intramolecular disulfide bridge and metal binding both impart exceptional stability to SOD1 and, unsurprisingly, loss of these factors drives misfolding and aggregation. However, reduction of the native Cys-57–Cys-146 disulfide has been putatively linked to the initiation, but not elongation, of amyloid-like fibril formation *in vitro*.[86,97] Disulfide-intact, but metal-free, SOD1 incubated at physiological pH and temperature can be induced to aggregate by disrupting noncovalent interactions with a chaotrope, but treatment with a reducing agent instead results in a 20-fold shorter lag period.[97] Disulfide bond reduction, while apparently dispensable for fibril formation *in vitro*, may specifically accelerate nucleation. Indeed, the presence of a small amount of disulfide-reduced wild-type or mutant SOD1 appeared to “recruit” disulfide-intact wild-type SOD1 into fibrils without the need for additional reducing agent.[97] The mechanism by which disulfide-reduced SOD1 facilitates fibril nucleation has not yet been demonstrated, although the requirement of Cys-57 and Cys-146 suggests that intermolecular cross-linking between these two residues may play a role.[97] It is also unclear whether *in vivo* SOD1 aggregation, which is not always amyloid-like, proceeds by elongation of nuclei.

The two free cysteines in SOD1, at positions 6 and 111, also appear to be involved in SOD1 oligomer assembly. *In vitro* aggregation of metal-free wild-type SOD1 coincides with a loss of free cysteines and oligomer formation is attenuated by mutations at either or both sites,[96,102] leading to the hypothesis that intermolecular disulfide cross-linking mediates oligomerization. However, more recent studies in mutant SOD1 transgenic mice show that aberrant disulfide linkages are present only in large-scale aggregates appearing late in the disease.[103,104] A secondary role for intermolecular disulfide cross-linking in aggregation is unsurprising given the reducing environment of the cytosol and may be due to “trapping” of SOD1 in a misfolded state after an initial destabilizing trigger, such as Zn^{2+} loss or altered conformational dynamics resulting from mutation.[87,88] Cell culture experiments reveal a key role for Cys-111 in the promotion of SOD1 oligomerization, as mutation of this residue, but not Cys-6, attenuated oligomer formation and protected cells from mutant SOD1-mediated toxicity.[105] It could be that the higher solvent accessibility of Cys-111 (and thus, increased susceptibility to aberrant intermolecular disulfide cross-linking) accounts for its particular importance in SOD1 oligomerization. However, recent investigations by our laboratory offer an alternate interpretation of this phenomenon. We recently confirmed earlier reports that Cys-111 forms a mixed disulfide with glutathione and showed that this modification is abundant in human tissue. Interestingly, Cys-111 glutathionylation triggers dissociation of both wild-type and FALS mutant dimers *in vitro*, thus promoting the first step in SOD1 aggregation.[105a] The characterization of intermolecular disulfide formation as a nonessential late event in oligomerization suggests that Cys-111 may primarily promote aggregation by its ability to be glutathionylated, a modification that destabilizes the native homodimer. Treatments used to prevent Cys-111-mediated SOD1 aggregation in previous cell culture

experiments,[105] such as addition of a reducing agent and overexpression of glutaredoxin, would remove the glutathione moiety in addition to reducing intermolecular disulfides. Therefore, further study would be useful to resolve the contributions of Cys-111 glutathionylation and intermolecular disulfide bond formation in oligomer formation.

An emerging question in the study of mutant-mediated SOD1 aggregation is the extent of involvement of the wild-type protein. Since most FALS patients with SOD1 mutations are heterozygous, recent studies have utilized transgenic mice expressing both human wild-type and FALS mutant SOD1 to more accurately recapitulate SOD1 behavior *in vivo*. Coexpression of $SOD1^{WT}$ exacerbates the disease phenotypes of $SOD1^{G93A}$[106,107] $SOD1^{G85R}$[108] $SOD1^{L126Z}$, and $SOD1^{A4V}$ mice,[92] hastening the appearance of cellular pathologies and shortening survival times (Fig. 5). The effect of the wild-type protein on $SOD1^{A4V}$ mice is particularly dramatic; even though FALS patients with this mutation exhibit particularly rapid disease progression, mice expressing only $SOD1^{A4V}$ do not develop motor neuron disease within their lifetimes.[48] The toxic effect of coexpressing wild-type protein may be a simple issue of protein copy number. An earlier study of G85R mice[51] did not find any effect of human wild-type coexpression on survival, but both $SOD1^{G85R}$ and $SOD1^{WT}$ were expressed at lower levels than in the more recent model.[108] The observation that mutant SOD1 toxicity depends heavily on protein abundance, while not surprising, is troubling since nearly all mutant SOD1 transgenic mice substantially overexpress the protein.[44] However, mice overexpressing $SOD1^{WT}$ alone, while exhibiting minor deficits in motor function, do not experience paralysis or die prematurely.[107] Thus, FALS mutants clearly possess intrinsic pathogenicity independent of gene dosage. Mutant-wild-type heterodimers and disulfide-linked aggregates containing both wild-type and mutant SOD1 have been observed,[92,108] suggesting that wild-type SOD1 is “recruited” into non-native oligomers by pathogenic mutants, possibly under conditions of oxidative stress. These studies present an incomplete picture of the role of $SOD1^{WT}$ in aggregation but highlight the need for further scrutiny of the physiological relevance of commonly used transgenic mouse models.

C. Toxicity of SOD1 Aggregates

While misfolding and aggregation has been convincingly linked to ALS pathogenesis, the species responsible for motor neuron death has not been identified. Insoluble inclusion bodies appear in brain stem and spinal cord coincident with symptom onset and accumulate progressively in the terminal stages,[109–113] leading to an initial belief that large-scale aggregates are themselves toxic. However, the ability to detect soluble misfolded SOD1 led to the discovery that these non-native forms are present from birth[91,114] and selectively enriched in motor neurons[89,91] of FALS transgenic mice. It thus appears that small misfolded SOD1 may be the actual toxic culprit(s), present throughout life but causing symptoms only when cells can no longer keep their deleterious effects in check. In such a scenario, assembly of soluble misfolded SOD1 into relatively inert inclusions is expected to be neuroprotective, a phenomenon that has been demonstrated for aggregation of Ab and huntingtin in Alzheimer's and Huntington's diseases, respectively.[115–117] However, the relative toxicities of small soluble oligomers and large-scale aggregates of SOD1 remain to be directly proven. Similarly, no consensus has yet been reached on the mode(s) by which non-native SOD1 kills cells. The evidence at present, though sometimes contradictory, identifies a diverse set of targeted organelles, signaling pathways, and other cellular processes. In the remainder of this chapter, we will discuss the various pathological processes occurring in ALS, with special attention to a potential causal role for misfolded and/or aggregated SOD1.

V. Motor Neuron Death in ALS: Apoptotic Versus Necrotic, Cell-Autonomous Versus Non-Cell-Autonomous

Classification of motor neuron death in ALS remains controversial. Spinal cord motor neurons of ALS patients and transgenic mice overexpress the pro-apoptotic BH3-only protein Bax,[118] and knocking out this protein in SOD1^{G93A} mice results in delayed disease onset.[119] However, activation of “executioner” caspases (caspase-3, caspase-6, and caspase-7) is not always seen[120–122] and the morphology of dying motor neurons is often uncharacteristic of apoptotic bodies.[123,124] The current model for neuronal death in ALS is the one that has characteristics of both apoptosis and necrosis, with “necrotic-like” and “apoptotic-like” processes dominating in different cell types and/or disease stages that have yet to be delineated.[121,125]

Another question pertaining to classification of cell death in ALS is the autonomy of this process in motor neurons. A cell-autonomous “dying forward” process was long assumed, in which dysfunction within motor neurons, independent of input from surrounding cells, leads to their death and a subsequent denervation of motor endplates. However, several studies using cell-specific expression of mutant SOD1 support a prominent role of non-neuronal cells in promoting cell death. The most striking evidence against cell-autonomous motor neuron death is the reported lack of ALS phenotype of transgenic mice expressing mutant SOD1 under a neuron-specific promoter.[126,127] Mice with supraendogenous neuron-specific expression do experience neurodegeneration but show different pathological changes compared to transgenics ubiquitously expressing FALS mutant SOD1. Symptom onset occurs later, is diffuse rather than focal, and lacks certain morphological hallmarks such as mitochondrial vacuolization.[128] Astrocytes, supporting cells that neighbor motor neurons, have also been proposed to turn deadly in ALS through defects in glutamate processing and other mechanisms (see below). While mutant SOD1-expressing astrocytes exert toxicity on motor neurons in coculture,[129] astrocyte-specific expression failed to cause motor neuron disease in mice.[130] Mutant SOD1 expression limited to microglia (phagocytic cells in the CNS) or Schwann cells, which myelinate motor axons, likewise produced no ALS phenotype.[44,131,132] Although mutant SOD1 in neurons, astrocytes, and microglia appears insufficient to provoke ALS symptoms in isolation, knockdown in these cell types using Cre–Lox systems or siRNA delays disease onset and/or progression in transgenic mice with ubiquitous expression.[133–135] Surprisingly, Schwann cell-specific knockout of mutant SOD1 was reported to accelerate disease progression.[136] Taken together, these studies highlight the importance of non-neuronal cells in ALS pathogenesis and progression and suggest that the primary site of dysfunction may not be the motor neuron itself (Fig. 6).

Interestingly, skeletal muscle-specific expression of mutant, and to a lesser extent, wild-type, SOD1 in mice was recently shown to cause early motor deficits, followed by neuromuscular junction (NMJ) dismantlement and late-onset motor neuron loss.[137] This result is surprising in light of previous studies showing no effect of mutant SOD1 knockdown in muscle[138,139] but is consistent with reports of muscular defects as the primary pathogenic events in ALS.[140,141] The ability of muscle-restricted expression of mutant SOD1 to provoke motor neuron degeneration, as well as the precedence of neuromuscular denervation in the disease course (Fig. 7), suggests a “dying back” model of ALS where loss of the neuronal cell body is not the initiating event. The primacy of the NMJ in ALS pathogenesis is further supported by studies showing that inhibition of pro-apoptotic machinery, while completely preventing motor neuron loss in mice, did not prevent denervation and offered little functional improvement or lifespan extension.[119,142,143] The mechanisms by which toxic signals are transmitted from muscle to NMJ to motor axons are unknown, but a recent study of SOD1^{G93A} mice noted increased retrograde axonal transport of proteins related to cellular stress and death.[144] Muscular overexpression of a

mitochondrial uncoupling protein, which disrupts ATP synthesis, was also sufficient to induce progressive NMJ dismantlement and motor neuron loss in mice.[145] Loss of compensatory reinnervation may also be involved in NMJ pathology. A skeletal muscle-specific microRNA was recently identified that slows disease progression in $SOD1^{G93A}$ mice by stimulating reformation of neuromuscular synapses with denervated muscles.[146] Growing support for a “dying back” hypothesis of ALS highlights the need for additional investigation of skeletal muscle as a primary site of pathology.

VI. ALS Comprises a Spectrum of Pathologies

On a subcellular level, ALS pathology is staggeringly complex and includes abnormalities in nearly all cellular compartments. Many of these are undoubtedly secondary effects or compensatory mechanisms for an initial dysfunctional “trigger,” the identification of which has remained elusive despite nearly 20 years of research on the molecular bases of ALS. We will review some of the more notable and well-studied pathological processes and discuss their relevance to the initial stages of disease, when therapeutic intervention may still be possible.

A. Excitotoxic, Inflammatory, and Oxidative Insults

In 1992, Rothstein *et al.* found defects in glutamate signaling in neuronal tissue from patients who died of ALS but not Alzheimer's and Huntington's diseases,[147] revealing a unique molecular basis for ALS. This phenomenon was later attributed to the selective loss of the astrocytic glutamate transporter EAAT2, which is crucial for prompt clearance of glutamate from the synaptic cleft after firing.[148] Both FALS and SALS patients, and mutant SOD1 mice, have decreased levels of functional EAAT2 protein (also known as GLT1) and increased circulating glutamate in the cerebrospinal fluid (CSF).[149–152] Work in transgenic mice confirms the importance of EAAT2/GLT1-mediated glutamate clearance to motor neuron health. Deletion of this gene is sufficient to induce progressive neurodegeneration,[153] and genetically encoded[154] or exogenously stimulated EAAT2/GLT1 overexpression[155] delays symptom onset in ALS mouse models. The mechanism(s) by which EAAT2/GLT1 is downregulated in ALS are not yet understood, and it is not clear whether decreased mRNA synthesis/stability is a factor. Postmortem spinal cord from ALS patients had normal EAAT2/GLT1 mRNA levels.[156] However, a later analysis of $SOD1^{G93A}$ mice using *in situ* hybridization and qRT-PCR revealed a substantial decrease in EAAT2/GLT1 promoter activity and transcript quantity concomitant with disease onset.[157] EAAT2/GLT1 is directly affected by several deleterious processes that occur in the ALS-affected CNS, suggesting that deficiency of its transport function and subsequent glutamate overload may be a secondary event in ALS pathogenesis. Caspase-3 activation (which is itself a relatively late-occurring phenomenon[158]) results in a truncated, inactive version of EAAT2/GLT1,[159] and oxidative damage to the C-terminus of EAAT2/GLT1 diminishes its ability to transport glutamate.[160–162] EAAT2/GLT1 expression in astrocytes is also subject to modulation by neuronal signaling, via activation of the transcription factors κB motif binding phosphoprotein (KBBP) by presynaptic axons.[157] Synapse loss and denervation in $SOD1^{G93A}$ mice results in decreased astrocytic KBBP and diminished expression of EAAT2/GLT1, revealing that the astrocytic glutamate transporter is downregulated in response to synaptic dysfunction.[157] Taken together, these lines of evidence show that deficient glutamate reuptake by astrocytes is induced by preexisting neuronal stress, which is further exacerbated by the resultant excitotoxicity (Fig. 8).

Prolonged hyperstimulation by glutamate induces death primarily by allowing persistent calcium influx through the Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, which are specifically enriched in motor neurons.[163–166] Excess Ca^{2+} floods into the mitochondria and overwhelms its natural buffering capacity,

triggering reactive oxygen species (ROS) production, disrupting protein homeostasis, and eventually activating the apoptotic machinery.[167,168] Cytosolic calcium overload further perpetuates itself by stimulating the opening of ryanodine receptors (RyR) on the endoplasmic reticulum (ER) membrane, allowing Ca^{2+} release from the luminal space into the cytosol.[169] AMPA receptor-mediated calcium influx also increases mutant SOD1 aggregation in cultured motor neurons[170] and mouse models of ALS,[171] which produces additional ER and mitochondrial dysfunction (see below). Glutamate excitotoxicity thus acts synergistically with protein aggregation and mitochondrial/ER dysfunction to stress motor neurons and activate apoptosis in SOD1-related FALS (Fig. 10). Motor neurons are selectively vulnerable to excitotoxic stress due to their abundance of AMPA receptors and low calcium-buffering ability.[172,173] Riluzole, the as-yet sole drug approved for the treatment of ALS, inhibits excitotoxic stress in neurons by slowing glutamate release and blocking AMPA receptors,[174–177] but confers a survival benefit of only few months.[178]

In addition to excessive glutamate, secreted oxidative, nitrative, and inflammatory factors also contribute to motor neuron stress and death in ALS, as illustrated by the cytotoxic effect of ALS patient CSF on healthy rat spinal cord cultures.[179] While it has become clear that neurons, astrocytes, and microglia are all capable of secreting pro-inflammatory cytokines and other inducers of cellular stress and death, the relative contribution of each cell type to the transmittance of stress signals in ALS is unresolved. Astrocytes expressing mutant SOD1 kill wild-type motor neurons in co-culture by secreting an unidentified soluble factor that activates the pro-apoptotic Bax protein.[129] Activated microglia, which are pathologic hallmarks in the CNS of ALS patients and mouse models,[180–184] release a host of inflammatory and proapoptotic factors. For example, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are activated, resulting in enhanced production of prostaglandins and nitric oxide (NO), respectively,[185] and programmed death signals such as the Fas ligand (FasL) and tumor necrosis factor-alpha (TNF-α) are released.[185,186] A great deal of crosstalk exists between the cellular responses to each individual cytokine. For example, COX-2 and iNOS are activated by TNF-α stimulation of astrocytes,[187] and the transcription of FasL in motor neurons is activated by NO.[186]

In cases of SOD1-related FALS, the mutant protein directly contributes to production of extracellular stressors. First, mutant SOD1 disrupts redox regulation of NADPH oxidase (Nox), a membrane-bound producer of extracellular superoxide, through a direct interaction with Rac1. Oxidizing conditions normally promote the dissociation of the SOD1–Rac1 complex and cessation of Nox activation, but mutant SOD1 remains bound to Rac1 and allows persistent superoxide production under these conditions.[188] Extracellular superoxide may then enhance neuroinflammation by stimulating microglia which are activated by ROS[189] (Fig. 10). Furthermore, mutant SOD1 may itself be a secreted factor that contributes to neurotoxicity. Mutations in SOD1 confer an affinity for the secretory vesicle proteins chromogranins A and B.[190] Localization of the mutant protein to secretory vesicles allows its transport from neurons and astrocytes to the extracellular space, resulting in activation of microglia and motor neuron death.[190] Neuroinflammatory and excitotoxic insults from microglia and astrocytes are thought to primarily affect disease progression rather than representing a primary trigger of disease.[191] However, some biochemical indicators of microglial activation, such their accumulation in the CNS and increased TNF-α production, are present prior to symptom onset in ALS mice.[186,192] Although relief of excitotoxic stress with Riluzole offers limited survival benefit, the combination of this or similar drugs with anti-neuroinflammatory agents may result in more satisfactory functional outcomes.

B. Dysregulation of Neurotrophic Factors and Axon Guidance Proteins

Neural networks are not static entities that remain stable indefinitely after development; rather, they require the continuous input of neurotrophic factors and axon guidance cues

secreted by glia and innervated muscle. Loss of survival-promoting neurotrophic signaling has therefore been proposed as a contributing factor to motor neuron demise in ALS. In support of this view, CNTF knockout produces progressive motor neuron death in mice[193] and exacerbates neurodegeneration in the $SOD1^{G93A}$ model.[194] Muscle-specific overexpression of glial cell-derived neurotrophic factor (GDNF) in the G93A mouse preserves NMJs and improves motor neuron survival,[195] suggesting therapeutic potential of neurotrophic factor supplementation. However, deficits in neurotrophic factors are not seen in ALS patients; to the contrary, GDNF and CNTF are upregulated in muscle, CSF, and postmortem spinal cord from ALS patients.[196–199] The over-abundance of these factors in symptomatic individuals suggests that their upregulation is part of a defensive response to existing pathology and is ultimately insufficient to halt disease progression. In line with this view, administration of CNTF[200] or brain-derived neurotrophic factor (BDNF)[201] showed no measurable benefit to ALS patients. It is possible that neurotrophic factors hold therapeutic potential if administered early and/or at intact NMJs (recapitulating the beneficial muscle-specific overexpression of GDNF reported by Li *et al.*[195]), but such a requirement likely precludes their usefulness to ALS patients.

VEGF and ANG, which are involved in maintenance of both neural networks and vasculature, have also been implicated in ALS.[202,203] VEGF, in particular, has received significant attention as a disease-modifying factor since the discovery that diminished VEGF expression in transgenic mice is sufficient to cause late-onset neurodegeneration.[204] Furthermore, ALS patients have decreased circulating levels of VEGF in CSF compared to healthy controls.[205] Mutant SOD1 directly contributes to VEGF deficiency through binding of the 3'-untranslated region (UTR) of VEGF mRNA, destabilizing transcripts and downregulating expression[206,207] (Fig. 10). Loss of VEGF function could also have a genetic basis independent of SOD1 mutations. Single-nucleotide polymorphisms in the VEGF promoter region were found to correlate with an increased risk of ALS,[26] although a recent meta-analysis of available genotype data restricts this effect to males.[208] Mutations in ANG were also linked to a small subset of ALS cases.[21–23] ALS-associated ANG mutations occur at functionally important residues involved in catalysis and nuclear localization[22] rather than hampering ANG expression, which is unchanged or even increased in ALS patients and mouse models.[209,210] The common functional consequence of ALS-associated ANG mutations appears to be an inability to promote neural connectivity and survival.[211]

The relative contributions of these proteins' neuroprotective and angiogenic properties to CNS health are unknown, but there is evidence that deficiencies in both functions could promote neurodegeneration. The neuroprotective effect of both VEGF and ANG in cell culture[90,204,211] gives strong evidence for neurotrophic action of these proteins independent of vasculature. However, decreased cerebral blood flow in ALS patients[212,213] and disruptions in the blood–spinal cord barrier of several mouse models[214] indicate that vascular dysfunctions are indeed present in ALS. In mouse models of ALS, overexpression of VEGF or its receptors, or administration of purified VEGF directly to the CNS, resulted in neuroprotection and prolonged survival,[215,216] leading to hope for VEGF administration as a therapeutic strategy. Restoration of ANG activity may also hold therapeutic potential. Further study is needed to explore this possibility and to resolve the molecular details of ANG and VEGF action in the CNS.

One possible mechanism for VEGF-mediated neuroprotection is its antagonism of the axon guidance protein Sema3A. Sema3A is a member of the semaphorin family of proteins, which guide axons to their targets during development and also play a role in the complex phenomena of neural network refinement and plasticity.[217] Sema3A is a secreted glycoprotein that acts as an axonal chemorepellent through binding of a neuropilin-1/plexin-A coreceptor complex, which triggers downstream cytoskeletal reorganization and axon

withdrawal.[218] These receptor components are expressed throughout adulthood in spinal cord motor axons, a sensitivity which allows them to avoid Sema3A-producing scar tissue during post-injury regeneration.[219–221] However, postnatal responsiveness to Sema3A may be a liability in individuals with SOD1 mutations. Terminal Schwann cells of SOD1^{G93A} mice release abnormally high levels of Sema3A into the NMJ before symptom onset,[219] which would be expected to induce axonal withdrawal from the synapse. Interestingly, VEGF also binds neuropilin-1, leading some to propose that it prevents denervation in ALS by competing with Sema3A for receptor binding.[219]

In addition to Sema3A, ALS patients show increased expression of other axonal chemorepellents, including ephrinA1 in motor neurons[196] and Nogo-A in muscle.[222] Muscle-specific overexpression of the secreted factor Nogo-A induced axon retraction from the NMJ in mice and higher Nogo-A expression in a subset of ALS patients correlated with disease severity.[223] Although the dysregulation of axonal guidance proteins is clearly correlated with ALS pathology, there is no strong evidence for causation. In fact, Nogo-A upregulation has been reported to occur in response to neuromuscular denervation,[224] not vice versa. However, overabundance of axonal chemorepellents at the NMJ following initial retraction would certainly inhibit compensatory reinnervation and may transform a minor insult into irreparable damage to the neuromuscular synapse (Fig. 9).

C. Axonal Structure and Transport Defects

The combination of polarity, high energetic demand, and extreme axon length (up to 1m)[225] makes axonal integrity paramount to motor neuron viability. Accumulation of neurofilaments, which maintain axonal diameter and structural integrity in motor neurons, is a long-recognized hallmark of ALS pathology in humans and mouse models and is thought to contribute to the selective vulnerability of long, large-caliber motor axons.[2,48,226–228] Neurofilaments consist of light (NF-L), medium (NF-M), and heavy (NF-H) subunits, in equal proportion, and their proper assembly is crucial to the maintenance and extension of vulnerable large-caliber motor axons.[229,230] Misassembly of neurofilaments due to over- or under-expression, mutation, or deficient transport of individual subunits results in their accumulation, further hindrance of axonal transport, and eventual motor neuron death.[231–236] Hyperphosphorylation of neurofilaments also contributes to defective transport by causing their detachment from motor complexes and promoting aberrant self-association.[237–240] In ALS, this phenomenon is attributable to overactivation of p38 MAP kinase and Cdk5, which phosphorylate NF-M and NF-H.[241–243] Neurofilament accumulation appears to be selectively deleterious to axons. Overexpression of NF-H causes sequestration of neurofilaments within the cell body and perikarya and markedly delays disease onset in mouse models of ALS.[244] In addition to relieving the axonal burden of neurofilament aggregates, thus facilitating transport, accumulated neurofilaments in the perikarya are thought to counter glutamate excitotoxicity by chelating excess calcium and/or binding the cytoplasmic domains of glutamate receptors.[245,246]

In addition to neurofilaments, axonal transport of many other cellular components is indispensable for motor neuron health and homeostasis. Transport between the cell body and neuromuscular synapse is mediated by the dynein/dynactin (retrograde) and kinesin (anterograde) motor protein complexes, which carry adaptor-bound cargo along axonal microtubules. Mutant SOD1 mice show presymptomatic defects in both anterograde and retrograde transport,[144,247,248] with a particular retardation in the trafficking of mitochondria[249,250] and cytoskeletal components such as neurofilament and tubulin subunits.[248] Mitochondria are normally enriched near the neuromuscular synapse to meet the high energetic and calcium-buffering needs of the firing axon.[251] Impaired anterograde transport may thus explain the early onset distal axonopathy observed in ALS mouse models. SALS patients show accumulation of mitochondria in proximal axons,[252] which is

further evidence for impaired anterograde transport as a fundamentally important mechanism of all ALS pathology. As with several other pathological processes in SOD1-related FALS, misfolding and aggregation directly impair axonal transport though aberrant interactions. Mutant SOD1 acquires the ability to bind motor complexes that are instrumental to both anterograde (kinesin-2[253]) and retrograde (dynein/dynactin[254,255]) axonal transport (Fig. 10). Mutant SOD1 also interacts directly with the 3'-UTR of NF-L mRNA,[256] resulting in decreased expression that is observed in both FALS and SALS patients.[257–259]

Because of its prevalence in human patients and early onset in ALS mouse models, dysregulation of neurofilament transport and metabolism became an early candidate for a common mechanism of ALS pathogenesis. While this hypothesis is attractive due to its apparent specificity for motor axons, evidence for a primary role of axonal neurofilaments in ALS is contradictory. While the aforementioned work by Couillard-Despres *et al.* indicates that reducing axonal neurofilaments dramatically delays ALS pathology,[244] a second study showed no benefit from sequestration of neurofilaments in the cell body and perikarya.[260] It may be that neurofilament dysfunction modifies neurodegenerative severity in ALS but is not sufficient to cause disease, a possibility that does not preclude defective axonal transport as a primary mechanism of pathogenesis. The early retardation of both anterograde and retrograde trafficking in motor axons undoubtedly initiates a spectrum of deleterious effects such as energetic deficiencies at the distal synapse and impaired neuromuscular communication. Further study is crucial to reveal the underlying mechanisms and consequences of axonal transport malfunction in ALS and to identify targets for therapeutic intervention.

D. Mitochondrial Dysfunction

Mitochondrial abnormalities such as swelling and vacuolization are pathological hallmarks in spinal cords of ALS patients and most transgenic mouse models,[49,107,121,261,262] leading to much interest in the mitochondrion's involvement in disease. Perturbed energy homeostasis and ATP deficits are observed in both SOD1^{G93A} mice and skeletal muscle biopsies from ALS patients.[263–268] One mechanism by which the FALS mutant G93A impairs cellular respiration is through a novel ability to bind cytosolic malate dehydrogenase, which disrupts the malate–aspartate shuttle.[269] Misfolded and aggregated SOD1 mutants also accumulate on the cytoplasmic face of the outer mitochondrial membrane and bind directly to the voltage-dependent anion channel (VDAC), depolarizing the membrane and disrupting the normal functioning of the electron transport chain (ETC)[266,270–272] (Fig. 10). ETC dysfunction is a notable convergence in the pathologies of sporadic and familial ALS, and ETC inhibition in SALS patients has been linked to mutations in mitochondrial DNA.[267,273,274] Mitochondrial genome instability has been proposed (controversially) to play a central role in the natural aging process,[275–279] which would offer a possible basis for the late onset of disease in SALS. Interestingly, SOD1^{G93A} mice and a subset of sporadic ALS patients are hypermetabolic[265,280] and administration of a high-fat diet modestly improved survival in mice.[265] The cause(s) and significance of hypermetabolism are unknown, as are the mechanisms by which aberrant metabolic states mediate toxicity in ALS. The surprising finding that metabolic dysfunction in skeletal muscle can provoke motor neuron death[145] suggests that mitochondrial defects may be central to the retrograde neurodegeneration seen in mouse models of ALS.

Mitochondria are also key players in the buffering of intracellular calcium, which in prolonged excess results in the activation of pro-oxidant and apoptotic factors such as nitric oxide synthase (NOS), phospholipases, and endonucleases.[281,282] ALS mice show a CNS-specific decrease in mitochondrial calcium loading capacity that precedes motor deficits.[283] Likewise, both ALS patients and mouse models have increased intracellular calcium

concomitant with mitochondrial damage.[283–285] It is not clear whether decreased mitochondrial buffering capacity precedes cytosolic Ca^{2+} overload or vice versa, since these processes reciprocally enhance each other[167,286] (Fig. 10). Depletion of mitochondrial calcium-buffering ability is particularly deleterious to neurons and skeletal muscle, whose normal functioning involves frequent influxes of calcium to generate action potentials. This, combined with the enrichment of mutant SOD1 in mitochondria of motor neurons,[266,271,272,287,288] muscle,[137] and astrocytes,[289] may account for the sensitivity of these cells to mutant SOD1-mediated toxicity. Disturbance of mitochondrial function may also directly cause cell death by activating the apoptotic cascade. Aberrant localization to the intermembrane space and matrix[288,290,291] disrupts the structural integrity of the organelle, resulting in release of the apoptotic trigger cytochrome *c*.[292] Misfolded SOD1 monomers and oligomers also provoke apoptosis by associating with the pro-survival factor Bcl-2. The normally anti-apoptotic Bcl-2 exposes a toxic BH3 domain upon mutant SOD1 binding, resulting in cell death and interference of synaptic transmission at the NMJ.[293–295] Given the presymptomatic, cell type-specific recruitment of mutant SOD1 to mitochondria,[271,287] dysfunctional changes in this organelle merit consideration as primary contributors to ALS pathogenesis.

E. Deficient Protein Quality Control

The presence of proteinaceous aggregates in spinal cords of FALS and SALS patients suggests that malfunction or overloading of protein quality control machinery is a common feature of neurodegeneration. The ubiquitin–proteasome system (UPS), in which ubiquitin-tagged proteins are targeted for proteasomal degradation, is one such mechanism of misfolded protein clearance. Degradation of misfolded mutant SOD1 proceeds via the UPS and impedes its functioning by sequestering proteasomal subunits and ubiquitin ligases such as Dorfin,[296–300] while proteasomal inhibition produces a reciprocal enhancement of SOD1 aggregation[93,301,302] (Fig. 10). Ubiquitin- and ubiquitin ligase-positive intraneuronal inclusion bodies are found in FALS mouse models[47,303] and postmortem spinal cord of SALS patients,[124,300,304–306] indicating UPS activity and sequestration in both forms of the disease. Studies of SOD1 mutant transgenic mice reveal proteasomal impairment in the disease-vulnerable spinal cord and brainstem only after the onset of symptoms[307,308]; so UPS dysfunction is unlikely to be an initiator of pathology in SOD1-related FALS. Interestingly, as disease progresses, constitutively active proteasomal components are replaced by inflammatory cytokine-responsive subunits to yield the inducible “immunoproteasome”, which degrades proteins into antigenic peptide fragments to be presented by the class 1 major histocompatibility complex.[307,309,310] Inhibition of immunoproteasome formation using a small-molecule anti-inflammatory agent shortens survival in a rat model of ALS,[311] but a more targeted genetic approach involving knockdown of the LMP2 immunoproteasomal subunit yielded no effect on survival in $SOD1^{G93A}$ mice.[312] The role of the immunoproteasome in ALS pathology is yet to be precisely determined, but its upregulation may be a response to glia-mediated inflammation in the CNS.[313,314]

Protein quality control by ER-associated degradation (ERAD) is also impaired in ALS, leading to stress signaling that can directly induce motor neuron death via activation of apoptosis. During synthesis and maturation of nascent proteins in the ER, misfolded species are cleared from the luminal space by the ERAD pathway (reviewed in Ref. 315). Dysfunction or overloading of ERAD results in accumulation of unfolded proteins and triggers the unfolded protein response (UPR).[316,317] Mutant SOD1 interferes directly with ERAD by binding to derlin-1, a transmembrane protein responsible for the translocation of misfolded proteins from the ER lumen,[318] as well as the ER-luminal chaperone BiP[319] (Fig. 10). Sustained ER stress in SOD1 mutant mice leads to the activation of ASK1, an apoptotic

protein kinase, and survival can be prolonged by ASK1 ablation.[318] Derlin-1 interaction was detected only after symptom onset,[318] but multiple triggers of ER stress are clearly present in ALS, as evidenced by presymptomatic UPR activation in SOD1 mutant mouse models[320] and upregulation of UPR components in SALS patients.[306,321,322] Furthermore, mutations in the UPR protein VAPB have been linked to some ALS cases.[20] Thus, ER stress may not be ruled out as a primary contributor to ALS pathogenesis nor may its involvement be limited to SOD1-related cases.

F. Aberrant RNA Processing

Malfunction and aggregation of two nucleic acid binding proteins was recently shown to be a common causal factor for some cases of both familial and sporadic ALS. Since the surprising observation that the 43-kDa *trans*-activating response region DNA-binding protein (TDP-43) is present in a majority of ubiquitinated proteinaceous inclusions in ALS and frontotemporal lobar degeneration (FTLD),[323,324] over 35 dominant mutations in TDP-43 have been linked to ALS.[309,325–343] TDP-43 is notably excluded from inclusions of patients with SOD1-related FALS,[344] perhaps an indication of divergent pathological mechanisms. However, it was recently shown that the small heat shock protein B8 (HspB8) is involved in clearance of both SOD1 and TDP-43 aggregates,[345] which is evidence that, despite differences in etiology, TDP-43 and SOD1-related ALS may respond to similar therapeutic approaches. Mutations in a second RNA/DNA-binding protein, fused in sarcoma (FUS) (also known as translocation in liposarcoma (TLS)), have also been linked to ALS and FTLD.[346–360] This common genetic basis for ALS and FTLD blurs the distinction between these disorders and may account for their co-occurance in some patients.[8]

The study of TDP-43- and FUS/TLS-related proteinopathies is a burgeoning field, as even the normal functions of these proteins were not well understood prior to the revelation of their roles in neurodegenerative diseases. Both proteins are widely expressed, predominantly nuclear proteins in healthy cells[361] and are involved in RNA processing events such as splicing and transcriptional regulation (reviewed in Ref. 362). Cytoplasmic aggregation and nuclear depletion are early, and perhaps independent, events in TDP-43-related ALS pathology[363–366] and are accompanied by proteolytic cleavage,[324,367,368] hyperphosphorylation,[367,369,370] and ubiquitination.[323,324] The combination of aberrant localization and posttranslational modification of TDP-43 in ALS raises the question of whether TDP-43 pathogenicity is a loss or gain of function. Does toxicity stem from the loss of normal TDP-43 nuclear function, or does cleaved, phosphorylated, or aggregated TDP-43 acquire cytotoxic properties? The TDP-43 C-terminal fragment is produced by caspase-3 cleavage[371–374] and increases in abundance as symptoms progress, suggesting that proteolysis of TDP-43 may be secondary to activation of apoptosis. Cytoplasmic inclusions stain negative for several known binding partners of TDP-43,[375] suggesting that aggregates do not exert toxicity by sequestering these components. However, the possibility that altered TDP-43 disrupts cellular homeostasis through novel aberrant interactions, as is the case with misfolded SOD1, has not been ruled out. FUS/TLS, while also aggregating in the cytoplasm,[354,357,360] appears to retain a more normal pattern of localization in the ALS-affected CNS, and neither phosphorylation nor cleavage is significantly correlated with disease.[354,357,360,370]

Intense study is under way to clarify the cellular functions of TDP-43 and FUS/TLS and the role of mutations in ALS and other neurodegenerative disorders. Interestingly, defective RNA processing has been noted previously in ALS and shown to cause EAAT2 deficiency,[376] a phenomenon that TDP-43 or FUS/TLS dysfunction may explain. Elucidation of the roles of TDP-43 and FUS/TLS in RNA metabolism has the potential to fill gaps in our understanding of numerous pathological deregulatory events in ALS.

VII. Concluding Remarks

The molecular biology of ALS is extraordinarily complex, and identification of the crucial initiating factors has remained elusive. However, a critical need exists for effective therapies to prevent loss of motor function and extend life. This effort should be focused on developing strategies for intervention at primary sites of dysfunction. In the case of SOD1-related FALS, protein misfolding and aggregation and calcium dysregulation drive many of the diverse pathological events in disease progression (Fig. 10) and should thus be considered prime candidates for therapeutic targeting. Mutant SOD1 transgenic mice will continue to be invaluable for mechanistic study of disease and development/evaluation of drug candidates. However, these models should be evaluated critically for relevance based on criteria such as copy numbers of wild-type and mutant SOD1 and presence of posttranslational modifications that affect stability.

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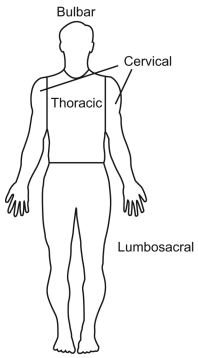


	Clinical presentation
Definite ALS	UMN and LMN signs in three regions (pictured left)
Probable ALS	UMN and LMN signs in two regions *AND* some UMN signs must be rostral to (above) LMN signs
Possible ALS	UMN and LMN signs in one region *OR* UMN signs alone in two or more regions *OR* UMN signs alone in two regions
Suspected ALS	LMN signs alone in two or more regions
All	Signs of degeneration spread within or between regions *AND* Lack of electrophysiological or neuroimaging evidence for other disorders which would explain observed degeneration

UMN signs: spasticity, hyperreflexivity *LMN signs: weakness, atrophy, fasciculation*

Fig. 1.
El Escorial criteria for diagnosis of ALS. UMN = upper motor neuron; LMN = lower motor neuron.

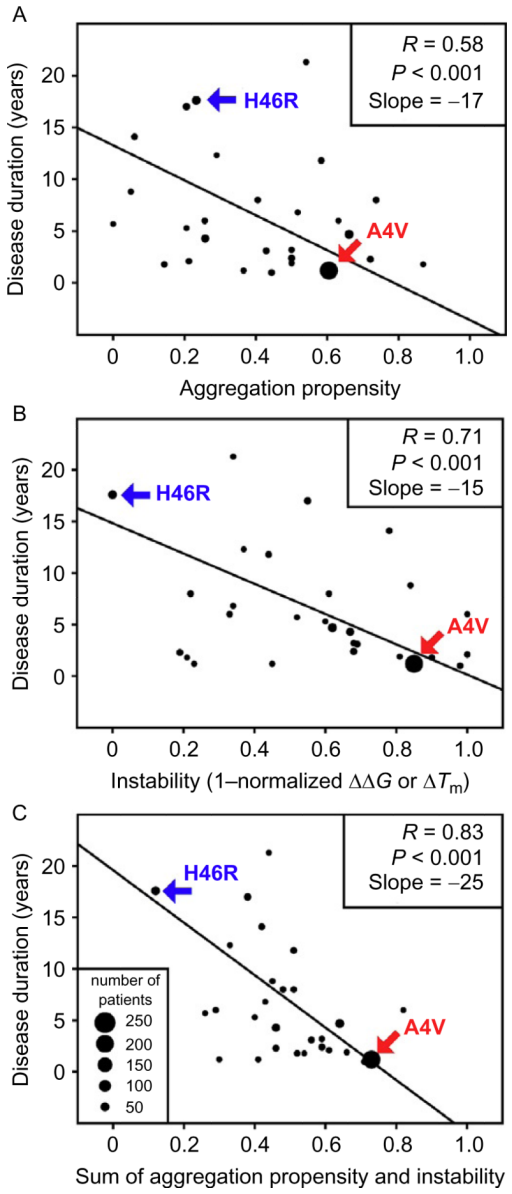


Fig. 2.

Protein instability and aggregation propensity correlate with shorter survival times in SOD1-related FALS. In all panels, data are weighted for linear regression analysis according to the number of patients for which survival data was available. (A) The aggregation propensities of FALS-causative SOD1 mutations are calculated using a rederivation of the Chiti–Dobson equation,[61] which was validated by comparison with available experimental data, and normalized such that the least and most aggregation-prone proteins have values of 0 and 1, respectively. (B) Protein instability is taken as 1 minus the change in free energy of unfolding or change in melting temperature of the mutant protein compared to the wild type (from published *in vitro* data). Instability values are normalized such that the most and least stable proteins have values of 0 and 1, respectively. (C) Normalized sums of aggregation propensity and protein instability values plotted against patient survival. From Wang *et al.*[60]

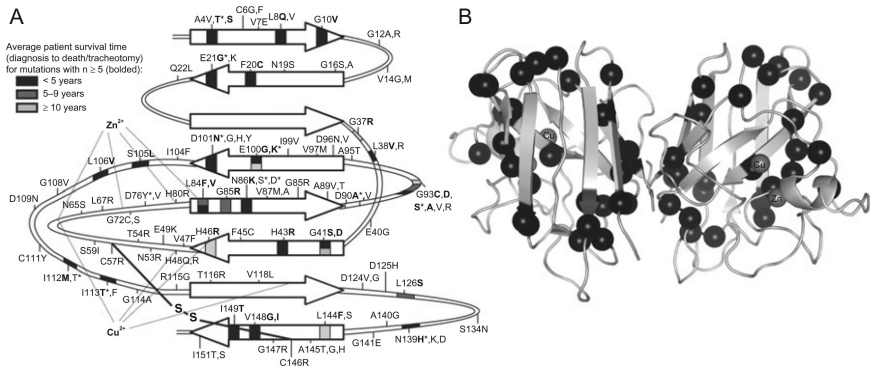


Fig. 3.

Location of FALS-causative mutations on the SOD1 structure. (A) Map of SOD1 secondary structure showing locations of FALS missense mutations, residues that coordinate Cu^{2+} (His-46, His-48, His-120, His-63) and Zn^{2+} (His-63, His-71, His-80, and Asp-83) ions, and the intramolecular disulfide bridge. Arrows indicate β-strands. Epidemiological data were taken from Refs. 45,60,70–73; mutations with survival data for at least five patients are bolded, and the residue position is shaded to indicate the corresponding average survival time. For positions with more than one $n \geq 5$ mutation, the upper color corresponds to the first mutation listed. (B) Crystal structure (PDB code 1spd) of fully mature (metal-bound, disulfide-intact) homodimeric SOD1 with positions of aggressive (survival time <5 years) mutations indicated by black spheres.

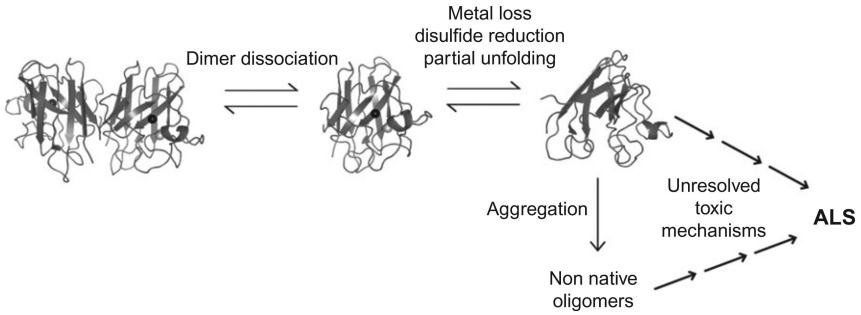


Fig. 4.
General mechanism of SOD1 aggregation.

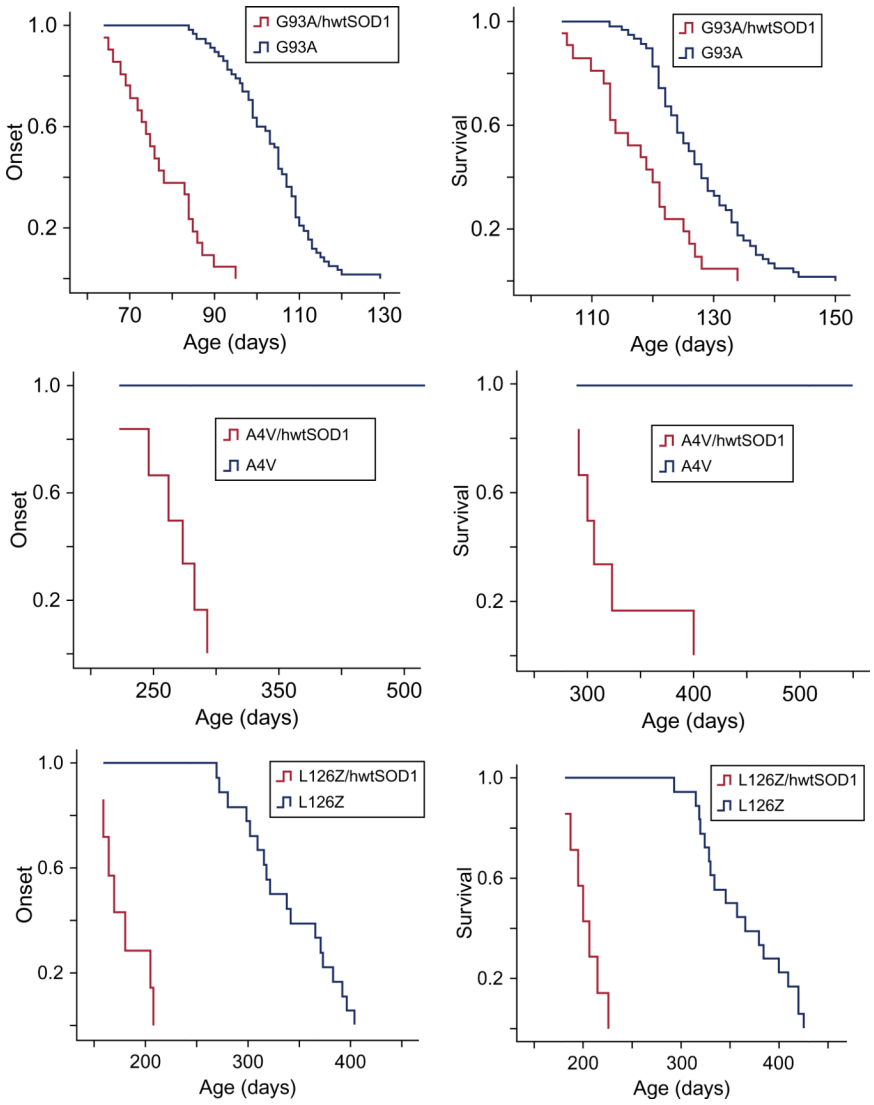
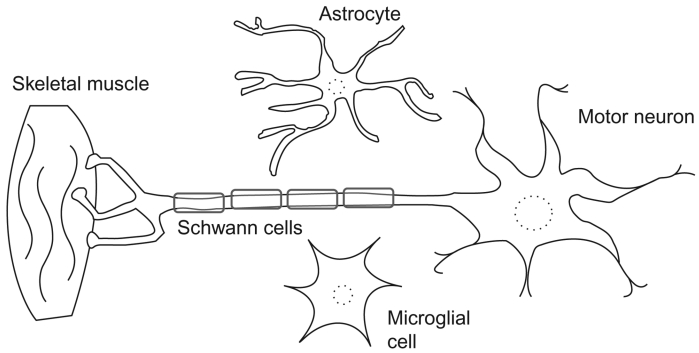


Fig. 5.

Coexpression of wild-type SOD1 exacerbates the phenotype of FALS mutant transgenic mice. Survival and symptom onset are plotted versus age for mice expressing G93A, A4V, and L126Z (truncation) mutants of SOD1 with and without coexpression of the human wild-type enzyme (hwtSOD1). From Deng *et al.*[92] Copyright 2006 National Academy of Sciences, USA.



Cell type	Restricted mutSOD1 expression sufficient to cause MND?	Effect of cell type-specific mutSOD1 knockout	Ref.
Motor neuron	Only when overexpressed	Delays onset, early progression	[126–128,133]
Astrocyte	No	Slows progression	[130,135]
Microglia	No	Slows progression	[131,133]
Schwann cell	No	Hastens late progression	[132,136]
Skeletal muscle	Yes	no effect	[137,138]

Fig. 6.

Motor neuron death in ALS is not cell autonomous. Table below the figure summarizes findings of studies using transgenic mouse models with tissue-specific mutant SOD1 expression or Cre–Lox/siRNA knockdown of ubiquitously expressed mutant SOD1.

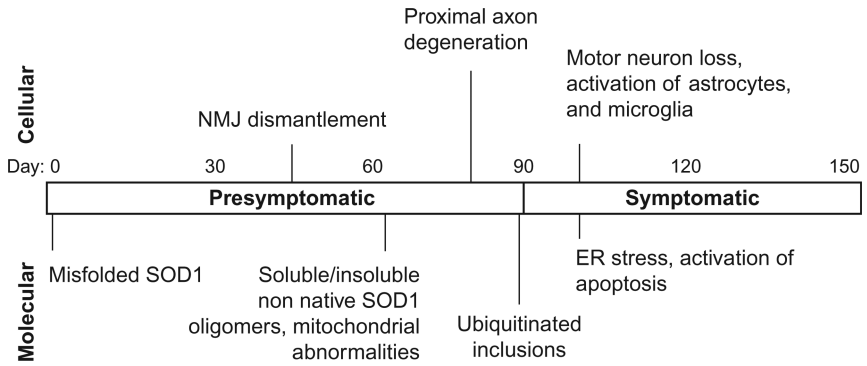


Fig. 7.

Timeline of molecular and cellular pathologies in transgenic mice ubiquitously expressing $SOD1^{G93A}$. The "symptomatic" stage denotes the period following initial onset of muscle weakness and wasting (80–100 days). Dates of pathology appearance taken from.[44,91,114]

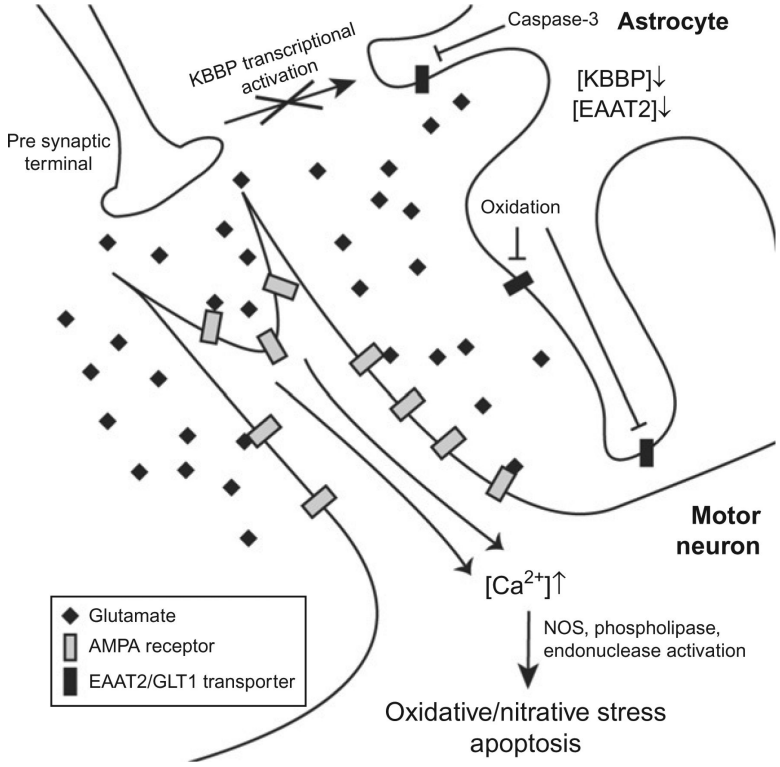


Fig. 8.

Glutamate excitotoxicity causes influx of Ca^{2+} to motor neurons and activates apoptosis. ALS patients and mouse models have decreased levels of the astrocytic glutamate transporter EAAT2/GLT1, which clears glutamate from the synapse after firing. Dysfunction in the presynaptic motor axon disrupts activation of the EAAT2/GLT1 transcriptional activator κB motif binding phosphoprotein (KBBP). Deficient EAAT2/GLT1, combined with inactivation of the transporter by oxidative damage and caspase-3-mediated proteolysis, results in persistent glutamate stimulation of the Ca^{2+}-permeable AMPA receptor, which is especially abundant in motor neurons. The resultant calcium influx activates several pro-oxidant and pro-apoptotic factors and prolonged Ca^{2+} excess results in motor neuron death.

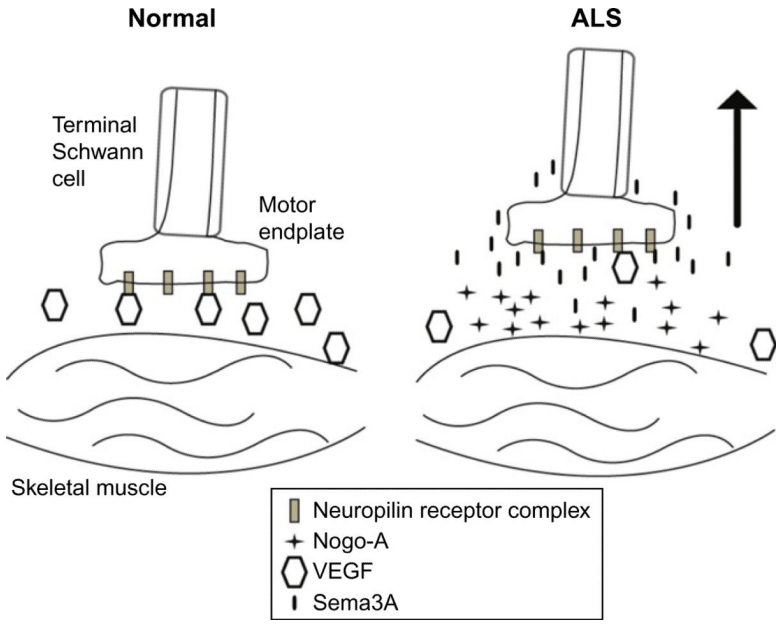


Fig. 9.

Aberrant expression of neurotrophic and axonal guidance factors at the neuromuscular junction of $SOD1^{G93A}$ mice. Expression of the neuroprotective vascular endothelial growth factor (VEGF) is decreased because of the binding of mutant SOD1 to the 3′-untranslated region of VEGF mRNA, while expression of the axonal chemorepellents Sema3A and Nogo-A is upregulated in terminal Schwann cells and muscle, respectively. Loss of trophic support and increased repulsive cues may induce withdrawal of the motor axon from the neuromuscular synapse.

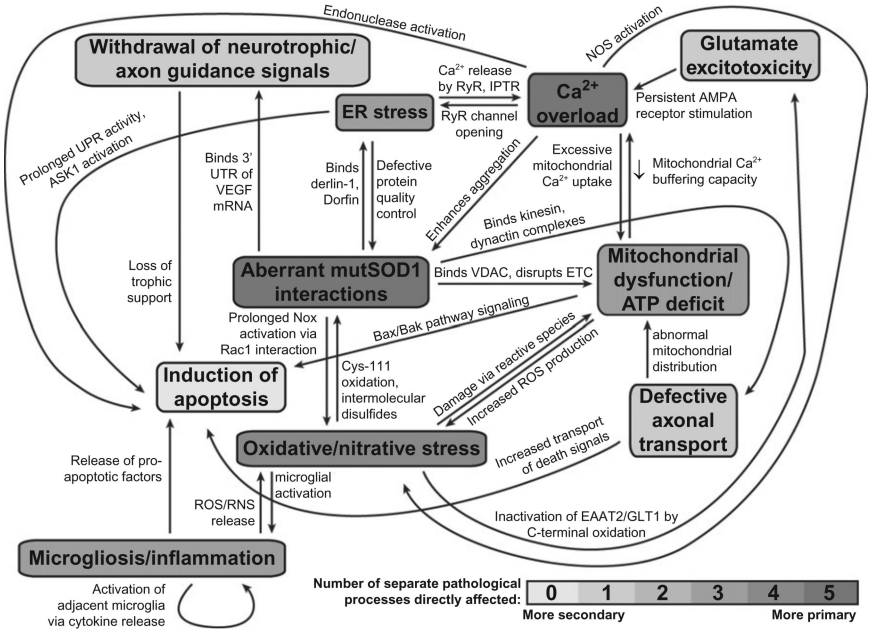


Fig. 10.

Diverse pathological processes in SOD1-related FALS are highly interrelated and many stem directly from SOD1 misfolding/aggregation and cytosolic calcium overload. Abbreviations: mutSOD1, mutant SOD1; UTR, untranslated region; VDAC, voltage-dependent anion channel; ETC, electron transport chain; UPR, unfolded protein response; ROS/RNS, reactive oxygen/nitrogen species.

TABLE I

Genetic Loci Associated with ALS

	Locus	Chromosome	Gene	Characteristics
Classical ALS	ALS1	12q22.1	Superoxide dismutase 1 (SOD1)	AD, adult onset
	ALS2	2q33	Alsin	AR, juvenile onset
	ALS3	18q21	Unknown	AD, adult onset
	ALS4	9q34	Senataxin (SETX)	AD, juvenile onset
	ALS5	15q15.1–21.1	Unknown	AR, juvenile onset
	ALS6	16q12.1–12.2	Fused in sarcoma (FUS)	AD, adult onset
	ALS7	20p13	Unknown	AD, adult onset
	ALS8	20q13.33	Vesicle-associated membrane protein-associated protein B (VAPB)	AD, adult onset
	ALS9	14q11	Angiogenin (ANG)	AD, adult onset
	ALS10	1p36	Tar DNA-binding protein (TARDBP)	AD, adult onset
	ALSX	X	Unknown	XD, adult onset
Atypical ALS	ALS/FTLD	9q21–22 9p13.3–21.3	Unknown	XD, adult onset
	ALS–PDC	17q21.1	Membrane-associated protein tau (MAPT)	AD, adult onset

AD, autosomal dominant; AR, autosomal recessive; XD, X-linked dominant; FTLD, frontotemporal lobar dementia; PDC, Parkinsonism-dementia complex.

Updated references for each locus at the ALS Online Genetics Database (http://alsod.iop.kcl.ac.uk).

Gray-shaded areas are alternated with unshaded rows to improve readability of the table