Review

Failure of protein quality control in amyotrophic lateral sclerosis

Edor Kabashi, Heather D. Durham *

Department of Neurology/Neurosurgery and Montreal Neurological Institute, McGill University, 3801 University St., Montreal QC, Canada H3A 2B4

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Abstract

The protein chaperoning and ubiquitin–proteasome systems perform many homeostatic functions within cells involving protein folding, transport and degradation. Of paramount importance is ridding cells of mutant or post-translationally modified proteins that otherwise tend to aggregate into insoluble complexes and form inclusions. Such inclusions are characteristic of many neurodegenerative diseases and implicate protein misfolding and aggregation as common aspects of pathogenesis. In the most common familial form of ALS, mutations in SOD1 promote misfolding of the protein and target it for degradation by proteasomes. Although proteasomes can degrade the mutant proteins efficiently, altered solubility and aggregation of mutant SOD1 are features of the disease and occur most prominently in the most vulnerable cells and tissues. Indeed, lumbar spinal cord of mutant SOD1 transgenic mice show early reduction in their capacity for protein chaperoning and proteasome-mediated hydrolysis of substrates, and motor neurons are particularly vulnerable to aggregation of mutant SOD1. A high threshold for upregulating key pathways in response to the stress of added substrate load may contribute to this vulnerability. The broad spectrum neuroprotective capability and efficacy of some chaperone-based therapies in preclinical models makes these pathways attractive as targets for therapy in ALS, as well as other neurodegenerative diseases. A better understanding of the mechanisms governing the regulation of protein chaperones and UPS components would facilitate development of treatments that upregulate these pathways in a coordinated manner in neural tissue without long term toxicity. © 2006 Elsevier B.V. All rights reserved.

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1. Introduction

The 2004 Nobel Prize for Chemistry was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation. In his Nobel lecture, Dr. Ciechanover stated: “With the multitude of substrates targeted and processes involved, it is not surprising that aberrations in the (ubiquitin–proteasome) pathway have been implicated in the pathogenesis of many diseases, among them certain malignancies, neurodegeneration, and disorders of the immune and inflammatory system” [1]. This review summarizes the current state of knowledge about the role of proteasome-mediated degradation in the pathogenesis of amyotrophic lateral sclerosis (ALS) and how impairment of the ubiquitin–proteasome system (UPS) might relate to inadequate stress responses in the affected neural cells. The UPS performs many homeostatic functions within cells including nascent protein folding, transport and degradation of proteins, signal transduction, cell growth and differentiation, and control of apoptotic pathways. The proteasome is also the major route for catabolizing misfolded proteins, preventing them from aggregating into insoluble complexes. Protein chaperones facilitate the restoration of functional protein conformation or target misfolded proteins for degradation. Thus, therapies that promote degradation of abnormal proteins may have broad applicability in neurodegenerative diseases.

2. Protein misfolding in ALS

Many neurodegenerative disorders, including ALS, have in common the sequestration of aberrant proteins into inclusions. The identity of the constituent proteins and morphology of the inclusions are pathognomonic of the disease. Proteins within these inclusions often are ubiquitinated, and protein chaperones and proteasomal subunits also may be sequestered [2–5]. Most
investigations of the role of the UPS in ALS have been conducted using experimental models of the most common familial form of ALS due to dominantly inherited mutations in Cu/Zn-superoxide dismutase (SOD1) [6]. Inclusions containing mutant SOD1 are found in motor neurons and sometimes surrounding astrocytes of patients [7,8] and transgenic rodent models [9,10], as well as in primary cultured motor neurons expressing several different SOD1 mutants [11].

Whereas SOD1WT is for the most part a soluble enzyme, disease-causing mutant proteins can be isolated in three different biochemical states: soluble in nonionic detergent, nonionic detergent-insoluble/SDS-soluble (up to 10%), and SDS-insoluble polymers (less than 0.5%) [12–14]. Large aggregates can be trapped by passing homogenate through filter traps [14] and visualized as inclusions by immunohistochemical labeling of tissue sections or transfected cells [2,9,14,15]. In transgenic mice, the amount of insoluble and high molecular weight mutant SOD1 is highest in tissue most vulnerable to disease, suggesting that cells in these regions process the mutant protein differently or inefficiently [12,14,16,17]. Similarly, the propensity of mutant SOD1 to form aggregates visible by microscopy depends on the cell type. The preferential vulnerability of motor neurons manifests in culture, mutant SOD1 forming inclusions in motor neurons of spinal cord cultures following gene transfer, but not in dorsal root ganglion or hippocampal neurons [11]. In various cell lines, treatment with proteasomal inhibitors [12] promotes the formation of inclusion bodies. Even mutant SOD1 that is immunolabeled diffusely may be in the form of small oligomers rather than the normal dimeric structure of SOD1WT [18], consistent with a significant fraction of the protein being detergent-insoluble even in the absence of distinct inclusions [13].

Whereas there is evidence that inclusions may protect cells from accumulated misfolded protein rather than being inherently toxic, they do reflect failure of the proteolytic system to rid cells of potentially harmful proteins [19]. In the case of mutant SOD1, there are many studies in which inclusions served as a marker of toxicity, although this by no means demonstrates they are the cause of toxicity: the load of protein aggregates correlates with motor neuron dysfunction in a transgenic mouse model [17]; cultured motor neurons with inclusions develop DNA fragmentation and chromatin condensation [11]; loss of viability correlates with formation of mutant SOD1 inclusions [11,12,18,20], and treatments that reduced formation of inclusions prolonged viability of mutant SOD1-expressing motor neurons [11,21] and Neuro-2A cells [22]. On the other hand, survival of differentiated PC12 cells transduced with mutant SOD1-YFP adenoviral vectors did not correlate with the number of inclusions (however, inclusions of SOD1WT also were observed in the study) [23], and targeting mutant SOD1 to mitochondria induced cell death in the absence of cytoplasmic inclusions, although oligomerization of mutant SOD1 in mitochondria was not excluded [24]. This is a complex issue that is influenced by the experimental paradigm (e.g., culture versus animal models, tagged versus untagged constructs, stable versus transient expression, exposure to additional stresses).

Application of more sensitive techniques to resolve smaller oligomeric forms of mutant protein in cells is needed, as these are the species considered most likely to be toxic [25].

Evidence points to a “toxic gain of function” conferring disease properties, with conformational abnormalities as the common property. Over 100 mutations in SOD1 cause disease, but have variable effects on enzymatic activity, and over-expression of SOD1WT failed to cause ALS-like disease [26,27]. Mutant SOD1 proteins, are catabolized by the proteasome in cells [12,28–30], and recombinant SOD1 mutants are good substrate for the 20S proteasome in vitro, particularly as metal deficient monomers lacking the intrasubunit disulfide bond [31].

Studies of recombinant proteins in vitro or transfected cultured cells subjected to oxidizing conditions show that dimer destabilization (SOD1 functions as a homodimer) and polymerization of monomeric SOD1 are promoted by disease-causing mutations, demetallation, disulphide reduction, and oxidative modifications [32–47]. Whereas these modifications alter solubility and promote aggregation in vitro, it is not known if the conformational alteration conferred by the mutation is sufficient to cause proteotoxicity in vivo or if other post-translational modifications contribute. In vivo, the major conformational changes of SOD1 in affected tissues reported are oxidation, protein nitration and 4-hydroxy-2-nonenal (HNE) modifications of amino acids [48–51]. Regardless, chaperoning and clearance of mutant SOD1 from the cell would prevent both toxicity and formation of inclusions and cells that accomplish this efficiently will be less vulnerable than those with less robust protein chaperoning and proteasomal capacity.

3. The role of the ubiquitin–proteasome system in protein turnover

Proteasomes are barrel-shaped protein complexes in the cytoplasm and nucleus responsible for degrading most nonvesicular proteins including short-lived regulatory molecules, damaged (oxidized, glycated, nitrated, etc.), incompletely translated, and mutant proteins in cells [3,52]. The ubiquitin–proteasome system works in partnership with molecular chaperones, highly conserved families of proteins important for protein folding and transport. Various forms of cellular stress can promote conformational changes that can alter the function of a protein and promote aggregation with like or other proteins [53]. Misfolding and aggregation are facilitated by molecular crowding in the intracellular milieu [54]. Chaperones aid partially folded or unfolded polypeptides to revert to their functional conformation, preventing their interaction with inappropriate partners and aggregation [55]. They are also involved in various cellular processes including nascent folding, transport and degradation of proteins, as well as signal transduction, cell growth and differentiation, and preventing apoptosis [56,57]. The most studied chaperones are the constitutively expressed heat shock cognate proteins (HSCs) as well as stress-inducible heat shock proteins (HSPs) [e.g., Hsp70(68,72,73), 40, 60, 90 and 110; the small HSPs: heme oxygenase, ubiquitin, Hsp25/27, αB-crystallin; the glucose
regulated proteins: Grp75,78,94] [58]. They protect cells by refolding or targeting abnormal proteins to the proteasome for degradation. HSPs are induced in response to a variety of stressful conditions (hyperthermia, oxidative stress, ischemia, calcium ionophore, trauma, exposure to toxic chemicals, mutant proteins, etc), a process termed the heat shock response. Cells that can mount a robust heat shock response are better positioned to survive the initial and subsequent insults. Fig. 1 summarizes the major pathways by which HSPs and proteasomes regulate protein degradation in cells.

3.1. Targeting aberrant proteins to the proteasome for degradation

Although HSPs have in common the ability to recognize and sequester misfolded proteins, Hsp70 and Hsp40, with associated proteins and ATP, can refold proteins to their functional conformation [59,60]. If proteins cannot be refolded, they are escorted to the proteasome for degradation. As a general principle, proteins are targeted to the proteasome by poly-ubiquitination, a process whereby ubiquitinating enzymes...
catalyze the covalent attachment of a chain of at least four ubiquitin molecules. Ubiquitin is adenylated and activated by binding of E1 (ubiquitin activating enzyme) in an ATP-dependent fashion, then transferred to an E2 ubiquitin conjugating enzyme. Then, HECT domain or RING-finger motif-containing E3 ligases mediate linkage of ubiquitin to lysyl side-chains of the substrates [61]. Whereas only one E1 mammalian gene exists, hundreds of E3 ligases provide a degree of specificity to ubiquitination of thousands of protein substrates. Polyubiquitination is facilitated by E4 enzymes, including C-terminus of Hsp70-Interacting Protein (CHIP) [62]. CHIP shuttles misfolded proteins from Hsp70 to the UPS, inhibiting Hsp70’s refolding activity through N-terminal TPR motifs and facilitating ubiquitination of substrate through its U-box (ubiquitin-ligase) domain [63]. CHIP promotes degradation of several disease-associated proteins including mutant SOD1 [64,65].

Linkage of at least four ubquitins via lysine 48 targets the substrates to the proteasome; however, monoubiquitination, or lysine 63-linked ubiquitin chains do not target proteasomal degradation, but serve other functions including signaling, endocytosis, vesicular transport, protein sorting and transcriptional regulation [66]. The process of ubiquitination is reversible by deubiquitinating (DUB) enzymes [67].

3.2. Proteasomal structure

The 26S proteasome is a complex of approximately 2.5 MDa in size comprised of a 20S catalytic core and two regulatory subunits that recognize and unfold substrates [52]. The 20S proteasome is a cylindrical unit consisting of 28 subunits (14 gene products), arranged as four heptameric staggered rings. The two outer rings contain the structural α-subunits (α1–α7), while the two inner rings contain two copies of the β-subunits (β1–β7), three of which (β1, β2 and β5) harbour the six active sites [68]. β5 cleaves preferentially after hydrophobic residues [chymotrypsin-like activity], β2 after basic residues [trypsin-like activity], and β1 after acidic residues [caspase-like activity, a.k.a. peptidyl-glutamyl peptide hydrolase (PGPH)]. Some of the β subunits are transcribed as proforms and need to be cleaved during the assembly process in order to be functional [69]. The assembly of the mammalian 20S proteasome is not well understood, but appears to be mediated by POMP or protoassemblin and Hsc73 [70,71]. Recently, two chaperones, PAC1 and PAC2, were identified that associate with proteasome precursors and promote proteasomal maturation [72]. The 19S is composed of 18 different subunits and is responsible for recognizing ubiquitinated substrates, unfolding them and chaperoning them towards the proteolytic cavity [73]. In yeast, the molecular chaperone, Hsp90, plays an important role in assembly of 20S and 19S complexes and maintenance of the resulting 26S proteasome [74].

In addition to general protein degradation, a key function of proteasomes is to generate peptides for antigen presentation by MHC class I. The cytokines LPS, TNF-α, and/or IFN-γ induce expression of three alternate catalytic subunits, β5i, β2i, β1i and components of a different regulatory subunit, 11S, also known as PA28 [75]. The inducible subunits replace the constitutive counterparts forming another complex known as the immunoproteasome [76]. Various intermediate/hybrid subtypes of proteasome also have been isolated including 20S core alone [77], 20S associated with one 19S and one 11S subunit [78–80] and 26S proteasomes with different β-subunits. Difference in subunit composition correlates with different hydrolytic activity, the initial cleavage site used and the peptide products generated. Although ubiquitination is the major signal for proteasomal degradation, the 20S proteasome is able to break down proteins in the absence of regulatory particles, particularly oxidatively damaged proteins [81].

3.3. Regulation of proteasome gene transcription

In comparison to regulation of immunoproteasome subunits, the molecular mechanisms regulating constitutive and stress-induced expression of proteasome subunits in mammalian cells are less well understood. In yeast, coordinated regulation of genes encoding components of the proteasomal and ubiquitin systems is achieved through binding of the transcription factor Rpn4 to a common proteasome-associated control element (PACE) [82,83]. Rpn4 regulates proteasome gene expression through a negative-feedback loop. The transcription factor is itself a proteasomal substrate, accumulating when proteasome levels are insufficient, and being quickly degraded when activity is restored [83,84]. No homolog of Rpn4 or its DNA-binding element has been identified in the mammalian genome. However, indirect antioxidants stimulate transcription of numerous genes encoding 20S, 19S and 11S proteasomal subunits, in addition to antioxidant/detoxifying enzymes, through the antioxidant response element (ARE), an action dependent on the Keap1-Nrf2 signaling pathway [85,86]. The transcription factor Nrf2 is negatively regulated by binding to Keap1 in the cytoplasm [87,88], which also promotes its degradation by the UPS. Thus, like Rpn4 in yeast, Nrf2 is maintained at low levels under low stress conditions by proteasomal degradation [89]. Upon exposure to electrophiles generated by xenobiotic or physiological stress, Nrf2 is released from Keap1, translocates to the nucleus and initiates transcription of a variety of stress response genes including phase II detoxifying enzymes and proteasomal subunits. Peroxisome proliferators also increase expression of proteasome genes, but independent of Nrf2 [90], indicating that proteasome levels can be altered through multiple pathways. Recently, Zil268was identified as a repressor of transcription of some proteasomal subunits [91]. Although in yeast, the major heat shock transcription factor (Hsf1) influences expression of ubiquitin–proteasome subunits (ubiquitin, an E3 ligase, and a ubiquitin conjugating enzyme) [92]. Hsf1 does not appear to regulate proteasome genes or activity in mammalian cells [93].

4. Methods of measuring proteasomal activity

Distinct catalytic activities have been assigned to each of the three active β-subunits and can be measured in cell/tissue homogenates using fluorogenic peptide substrates specifically
hydrolyzed by chymotrypsin-like (β5), trypsin-like (β2), and PGGH/caspase-like (β1) activities [94–96]. This technique measures the ability of proteasomes present in the tissue extract to catabolize an additional substrate load (fluorogenic substrates) and can be expressed relative to a housekeeping protein in the sample (total proteasome activity) or to levels of specific proteasome subunits (specific activity). Proteolytic activity of both 20S core alone and 20S with regulatory particles attached (26S) is measured. The relative contribution to total activity will depend upon the abundance in the tissue and the method of tissue preparation; i.e., whether 26S remains intact or 19S/11S detach. Addition of glycerol and ATP to the buffer preserves the interaction of 20S core with regulatory particles [97].

To obtain measures of proteasome activity in vivo, fluorescent reporter proteins have been constructed. Kopito’s group coupled green fluorescent protein (GFP) to a short degron, CL1 [ACKNWFSLLSHFVIHL] (GFP”), which promotes its poly-ubiquitination and degradation by the 26S proteasome [98]. Dantuma’s group has constructed two different ubiquitin-tagged GFP substrates. First, the ‘N-end rule’ substrate, Ub-R-GFP, in which the N-terminal arginine of GFP is recognized by the E3 Ub1 after cleavage by deubiquitinating enzymes. In the second construct, UbG76V-GFP, an N-terminally linked ubiquitin serves as the acceptor for polyubiquitination, but the G76V substitution prevents de-ubiquitination, thereby promoting efficient degradation [99,100]. All of these reporter proteins have short half-lives because of their efficient degradation by the UPS and accumulate when proteasome activity is inhibited; thus increase in GFP fluorescence is a measure of reduced ubiquitin-dependent proteasomal activity. Lines of mice carrying the UbG76V-GFP [101] or GFP-CL1 (GFPdgn) [102] transgene have been generated to provide a readout of proteasome function in vivo. The ability to monitor proteasome function in individual cells without lysing tissue is more meaningful than having a measure of activity in homogenerate. However, this in situ method is not completely straightforward. Only ubiquitin-dependent proteolysis is monitored (i.e., 26S activity) and the GFP reporter can itself aggregate and compromise chaperoning capacity if expression levels are too high [103]. The other issue is that rather high level inhibition of specific proteasome activity, as measured by fluorogenic substrates in homogenates, is required to observe substantial increase in GFP reporter fluorescence [99,101,104]. At such levels of inhibition represent a threshold of function that is only compatible with life over the short term, the reporters may only monitor end-stages of pathogenesis. On the other hand, biochemical measures of proteasome activity do not provide sufficient information on functional impairment or cell type differences. A combined approach using both biochemical and in situ techniques is more informative.

Native gel electrophoresis is useful to examine the subunit composition of proteasomes. Separation of 20S, 26S, precursors and unassembled subunits can be accomplished by the 2-D Coomassie-Blue native gel method; tissue lysates are run in the first dimension on Blue native gels, then strips are placed horizontally over denaturing SDS-polyacrylamide gels for separation of the complexes into subunits in the second dimension [105]. The native gel electrophoresis method described by Finley and colleagues is enriched with ATP and glycerol and favours retention of the 26S complex [97]. In gel activity assays using fluorogenic substrates provides a crude determination of the proteolytic activity in the tissue samples [97]. These are powerful techniques to evaluate proteasome composition in small tissue samples.

5. Proteasome activity and structure in ALS

Because treating cells expressing mutant SOD1, but not wild-type (WT) SOD1, proteins with proteasomal inhibitors promotes insolubility and accumulation of the mutant protein as well as formation of microscopically visible inclusions, it would be logical that proteasomal function would be compromised in cells and tissues that exhibit inclusions without exposure to inhibitors.

5.1. Interaction of mutant SOD1 with UPS components

Inclusions in spinal cord of several mutant SOD1 transgenic mouse models often are labeled by antibodies to members of the ubiquitin–proteasome pathway [2]. There is biochemical evidence showing that mutant SOD1, but not WT is poly-ubiquitinated both in neuronal and non-neuronal cells and is subsequently degraded by the 26S proteasome [29,36]. Several E3 ligases can poly-ubiquitinate mutant SOD1 in vitro. The E3 ligase, dorfim interacts with mutant SOD1 and its overexpression decreases mutant SOD1 toxicity [29]. Two other E3 ligases, CHIP and NEDL1 also can interact with and ubiquitinate mutant SOD1 [65,106,107]. Using microfluorometric imaging techniques, Matsumoto et al. showed stable association of transfected β5i (LMP7) proteasomal subunits with mutant SOD1 inclusions in differentiated PC12 cells [18].

Collectively, these data show that mutant SOD1, but not WT SOD1 proteins are catabolized by the proteasome and that biophysical species with altered solubility are precursors of larger inclusions. Under most circumstances cells are capable of handling mutant SOD1 proteins sufficiently to prevent them from exerting toxicity and/or being sequestered into inclusions. Even motor neurons develop and function into adulthood. However, under circumstances of increased physiological or environmental stress or compromise of protective mechanisms with aging, the UPS may become overloaded and impaired.

5.2. Measures of proteasomal activity in cell and mouse models of fALS

Several studies have measured total proteasomal enzyme activity by hydrolysis of fluorogenic substrates in cell lines transfected with mutant SOD1 with mixed results. Total proteasomal activity was decreased [36,108,109], increased [110,111] or unchanged [112]. Differences in physiological properties of clonal cell lines, including ability to upregulate proteasomes or other protective mechanisms may have contributed to disparate measures of activity.

In our laboratory, total and specific proteasomal activities were measured in homogenates of tissue from SOD1G93A and
SOD1<sup>WT</sup> transgenic mice and their non-transgenic littermates [16]. Both total and specific chymotrypsin-like, trypsin-like and caspase-like activities were significantly reduced in lumbar spinal cord of SOD1<sup>G93A</sup> transgenic mice, the tissue that is ultimately most affected pathologically, but not in the remainder of the spinal cord or in liver. Chymotrypsin-like activity was significantly impaired as early as 45 days postnatal, with all three activities reduced by about 50% at day 75 [16]. The early decline in chymotrypsin-like activity corresponds to a reduction in 20S β5 subunits, but not 20S α, 19S or 11S subunits (Fig. 2A, B and D). Subsequently, 20S α levels also decreased specifically in lumbar motor neurons (Fig. 2C), indicating that general levels of proteasome are reduced in affected neurons during the symptomatic phase of the disease [16,113]. These

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**Fig. 2.** Impairment of both specific proteasome activity and altered proteasomal subunits in lumbar spinal cord of SOD1<sup>G93A</sup> transgenic mice. (A,B) Chymotrypsin-like, trypsin-like and caspase-like proteasomal activities were measured by hydrolysis of exogenous fluorogenic substrates in homogenates of lumbar spinal cord of SOD1<sup>G93A</sup> transgenic mice, SOD1<sup>WT</sup> transgenic mice and their nontransgenic littermates at 45 days postnatal, 75 days or at symptomatic phase (approximately 120 days of age). Original data showing decrease in total and specific activities in lumbar spinal cord but not thoracic spinal cord or liver is published in Kabashi et al. [16]. In this figure, means for each of the three activities in transgenic tissues relative to littermate were averaged to obtain a measure of overall proteasome hydrolytic capacity. (A) Total proteasome activities (normalized to actin) and (B) specific proteasome activities normalized to level of 20S α structural subunits. (C) Whereas levels of 20S α are unchanged at early stages of decreased proteasome activities, immunolabeling of 20S α subunits was dramatically reduced, specifically in motor neurons relative to the surrounding neuropil at later stages. Shown is immunolabeling with antibody to α subunits of the 20S proteasome core from Affiniti and antibody specific to human SOD1 from Sigma-Aldrich. Similar data were published previously in Kabashi et al. [16], and Cheroni et al. [113]. (D) Overall levels of 20S α in homogenate of lumbar spinal cord of symptomatic SOD1<sup>G93A</sup> and nontransgenic littermates are similar, but the amount of 20S β5 subunit (associated with chymotrypsin-like activity) is markedly reduced (in parallel with decreased chymotrypsin-like activity and before gliosis [16]. This is partly compensated for by increased expression of β5i indicating a shift to immunoproteasomes in concordance with the presence of activated microglia in the region. Increase in immunoproteasome was previously reported by Kabashi et al. [16], Cheroni et al. [113] and Puttaparthi et al. [114]. Data for Fig. 2 contributed by Edor Kabayashi and David Tayor and by Dr. Jeffrey Agar, currently at Brandeis University.
findings point to the necessity of conducting biochemical measurements on specific regions of spinal cord in disease models rather than pooling homogenates of whole spinal cord. Measures of proteasome activities in whole spinal cord homogenate from mutant SOD1 mice are unchanged or increased relative to controls [113,114] and Kabashi et al. (unpublished data).

Astrocytosis and gliosis are prominent features of spinal cord pathology in both ALS patients and transgenic mouse models and are fulminant by symptomatic age (120 days in the high copy number SOD1G93A mouse line). This is reflected in an increase in inducible components (β5i and IIS) of the immunoproteasome in symptomatic mice, substituting for constitutive 20S β subunits and 19S subunits [16,113,114] (Fig. 2D).

5.3. Relevance of proteasomal dysfunction to ALS pathogenesis

The above findings demonstrate compromise of proteasomal catabolic capacity in vulnerable tissues in a transgenic mouse model of familial ALS. However, how that relates to disease pathogenesis in the mouse model and in familial and sporadic ALS patients requires further work. Measures of proteasomal activities in homogenates of lumbar spinal cord of SOD1G93A mice are reduced early in the disease process, but the cell types involved and the functional consequences remain to be elucidated. Bendotti’s group is attempting to answer this question by crossing SOD1G93A mice with UbG76V-GFP transgenic mice. As discussed above, a caveat of the GFP reporters of proteasomal function is that significant loss of activity may be required before GFP accumulates to readily visible levels ([99,104] and how long various spinal cord cell types live with this level of functional impairment is not known.

Studies in cell culture also support a significant role for proteasome dysfunction in mutant SOD1 toxicity. Formation of mutant SOD1-containing inclusions correlates with cell death in primary cultured motor neurons [11]. Aggregation and toxicity of mutant SOD1 are promoted by treating cells with proteasome inhibitors [12,29,30]. In time lapse studies in PC12 cells, levels of mutant SOD1 and a ubiquitin-GFP reporter of proteasome activity increased before inclusions formed [18]. These events correlated with subsequent cell death. On the other hand, proteasome inhibitors increased the number of aggregates formed but not cell death in PC12 cells expressing mutant and wild type SOD1-YFP constructs [23]. Also, motor neurons in organotypic spinal cord cultures prepared from SOD1G93A mice did not show a preferential vulnerability to proteasome inhibitors compared to those in cultures from non-transgenic mice [115].

Another issue is the mechanism underlying reduced proteasome activity in lumbar spinal cord of SOD1G93A mice. The early decrease in chymotrypsin-like activity appears too great to be explained only by substitution of immunoproteasome for constitutive proteasome or by changes in the cell type composition of the tissue because of increased presence of activated microglia and astrocytes, although these events do occur in late pre-symptomatic and symptomatic stages [16]. Reduction in 20S β subunits without decrease in α subunits is puzzling considering the proteasome ring structure. Preliminary data from our laboratory show no significant reduction in tissue mRNA levels or an increase in unassembled α subunits. Other possibilities being explored are post-translational modification of proteasomal proteins or substitution of other subunits. Depletion of proteasomes by sequestration in inclusions or choking of the proteasome by inclusions are unlikely mechanisms or [104].

Why does proteasomal impairment occur preferentially in the most vulnerable tissue (lumbar spinal cord), but not more generally? Although activities of proteasomes in spinal cord may be relatively low compared to other tissues [30], proteasome levels are normally high in motor neurons relative to the surrounding neuropil [16,116] (Fig. 2C). The high level expression of proteasomes and the high sensitivity of motor neurons to proteasome inhibitors [36] would indicate that these cells normally have a large substrate load. Part of this may be due to neurotransmitter input because blocking AMPA glutamate receptors both prolongs viability of cultured motor neurons expressing mutant SOD1 and reduces formation of inclusions [117]. Molecular crowding [54] or an intracellular milieu favoring hydrophobic conformations of mutant SOD1 or other proteins could also contribute [45].

How could impaired proteasome function contribute to disease pathogenesis? Apoptosis is promoted by accumulation of abnormal proteins and through depletion of HSPs that have direct inhibitory effects on apoptotic pathways in addition to their protein chaperoning activity [3]. The proteasome is the major pathway for degradation of transcription factors and other short-lived regulatory proteins, thus proteasomal inhibition could alter transcription of multiple gene families including those that promote cell death [118]. Another important function of proteasomes is degrading misfolded proteins shuttled into the cytoplasm from the endoplasmic reticulum (ER). ER stress results from disturbance of ER calcium homeostasis or an imbalance in the amount of misfolded protein and ER chaperoning capacity; both ER and Golgi networks are disrupted in ALS and experimental models of mutant SOD1, implicating multiple compartments in failure of protein quality control [119]. Proteasome inhibitors also disrupt mitochondrial homeostasis [120,121]. Motor neurons in ALS patients and in SOD1G93A and SOD1G37R transgenic mice do exhibit mitochondrial abnormalities [122] and the relationship between mitochondrial and proteasomal dysfunction in ALS deserves investigation.

5.4. Is the UPS a therapeutic target for ALS?

Either an increased generation of misfolded proteins or decreased rate of hydrolysis could cause accumulation of aggregation-prone or toxic proteins. Although increasing proteolytic capacity would seem to be a rational approach, inappropriate increase in proteasome activity could have negative consequences. A clinical trial of recombinant human ciliary neurotrophic factor (CNTF) in ALS patients was halted because of dramatic loss of lean body mass [123]. CNTF reportedly increases 20S proteasome mRNA and proteolytic activity [124]. A kinder gentler way of assisting cells to cope
with their proteotoxic load could be through stimulation of the Keap1-Nrf2 pathway whereby genes encoding multiple protective phase II antioxidant enzymes as well as proteasomal subunits are coregulated through ARE. Such treatments might also boost the antioxidant capacity of motor neurons, which normally have low levels of reduced glutathione [125]. Interestingly, Nrf2 mRNA was reduced in spinal motor neurons of two fALS cases [126] Activation of Nrf2 in astrocytes can indirectly protect neurons, including motor neurons [127,128]. That dietary factors stimulate these pathways has implications for prevention as well as treatment [129].

6. Role of Protein Chaperones in ALS

6.1. Reduced protein chaperoning activity

Protein chaperones also play major roles in maintaining protein quality control, working in complementary fashion with antioxidant enzymes and proteasomes. The capacity for both proteasomal protein degradation and protein chaperoning are preferentially reduced in lumbar spinal cord of mutant SOD1 transgenic mice, but not in tissues resistant to disease. In two lines of mutant SOD1 transgenic mice (SOD1G93A and SOD1G85R), chaperoning capacity was reduced in the lumbar region of the spinal cord prior to the onset of symptoms [21,130]. Why chaperoning activity is diminished is not known, but logically would involve increased load of misfolded proteins without increased levels of HSPs/HSCs to maintain chaperoning function. The following evidence supports direct interaction: Hsp70 was detected in the insoluble fraction of spinal cord from SOD1G93A, but not SOD1WT, transgenic mice and in spinal cord cultures prepared from E13 SOD1G93A mice [13]. Similarly, the small HSPs Hsp25 and αB-crystallin cofractionated with mutant SOD1 in a transgenic mouse expressing SOD1 with the copper-coordinating histidines mutated [131]. This could result from association with SOD1 or other misfolded proteins. Post-translational modification of Hsp70 by HNE has also been reported in these mice and could affect its chaperoning activity or solubility [50]. Also, HNE-modified proteins are potent inhibitors of the proteasome complex [132]. Direct association of HSPs with mutant SOD1 is indicated by presence of Hsc70 in inclusion bodies in spinal cord of G93A and G85R SOD1 transgenic mice [2] and by transient association of Hsp70 with mutant SOD1 inclusions in transfected cells [18]. Urushitani et al. reported transient association of Hsp/Hsc70 with SOD1 and CHIP [65]. However, Hsp70 was not detected in inclusion bodies from mutant SOD1 transgenic mice and was rare in ALS patients [2,133].

6.2. Is increasing levels of HSPs protective in models of fALS?

Over-expression of Hsp70 in cultured motor neurons significantly decreased mutant SOD1 toxicity [21], although this chaperone alone did not significantly affect disease in transgenic mice [134]. However, upregulation of multiple chaperones is more likely to have therapeutic benefit. A Hsp70 and Hsp27 were more protective in combination than individually in dorsal root ganglion neurons transduced to express mutant SOD1 [135]. Arimoclobol, a coinducer of heat shock gene transcription, improved hind limb muscle function, delayed motor neuron death, and led to a 22% increase in lifespan of SOD1G93A transgenic mice [136]. In our primary culture model of fALS, co-expression of a constitutively active form of the major heat shock transcription factor, Hsf1, or treatment with geldanamycin (also known to activate Hsf1) dramatically induced expression of Hsp70 and Hsp40 and was highly protective [133,137].

Despite the protective properties of HSPs, motor neurons have a high threshold for inducing a heat shock response to various stressors, including heat shock [133,138]. Nonsteroidal anti-inflammatory drugs (salicylate, cyclo-oxygenase 2 inhibitors), which increase HSP expression by increasing Hsf1 binding to DNA in response to stress, are effective inducers of Hsp70 in cultured astrocytes, but not in motor neurons in either the absence or presence of heat shock or disease-related stressors [139]. Similarly, in mutant SOD1 transgenic mice, HSPs are unchanged or decreased in motor neurons, but small HSPs (Hsp25 and αB-crystallin) are upregulated in reactive astrocytes [131,133,140–142]. Although upregulation of small HSPs is characteristic of reactive glia in mice, this was not observed in spinal cord of ALS patients [133]. Understanding the mechanisms whereby some cells upregulate HSPs in response to stress and others don’t will aid in development of compounds effective in neurodegenerative disease.

6.3. Regulation of HSP gene transcription

Eukaryotic expression of HSPs is mediated by binding and activation of heat shock transcription factors (HSF) to heat shock elements (HSEs) on HSP promoters [143–145]. Environmental and physiological stresses activate transcription of HSP genes in mammals largely through Hsf1 [143]. In the most accepted model, Hsf1 is sequestered in the cytoplasm bound to a multichaperone complex including Hsp90 and Hsp70. Upon stress, misfolded proteins compete for HSPs, releasing Hsf1 to translocate to the nucleus and bind as a trimer to HSE in HSP promoters. Subsequent steps are required to activate Hsf1 including phosphorylation of key residues in its regulatory domain, and release of inhibitory chaperone complexes [143,144,146]. Redox-dependent activation of Hsf1 through oxidation of cysteine residues could also promote trimerization and DNA binding [147].

Another HSF, Hsf2, is an important developmental regulator [148], but might also be involved in the heat shock response in association with Hsf1 [149]. Like many transcription factors, Hsf2 is a proteasomal substrate and accumulates with proteasome inhibition [150]. Treatment of cultured motor neurons with proteasome inhibitors causes both nuclear accumulation of Hsf2 and expression of Hsp70 [133]. It is not clear to what extent Hsf2 is responsible, but this pathway is potentially an alternative therapeutic target to enhance HSP production.
7. Conclusions

Matching levels of protein chaperones and the UPS to the substrate demand in times of stress is highly important to maintain homeostasis. Some cells, some tissues and some individuals do that better than others [151]. In models of familial ALS, both proteasome and chaperoning function are abnormal in the tissue most vulnerable to the disease process. Whether this is the case in familial and sporadic ALS patients is under investigation. The broad spectrum capability for cytoprotection makes these pathways attractive as targets for therapy in ALS, and other neurodegenerative diseases. However, HSPs also can be toxic [57] and the mechanisms governing their regulation at the transcriptional and post-transcriptional levels are highly complex. A better understanding of these mechanisms, development of treatments that upregulate these pathways in a coordinated manner, and use of preclinical test models that evaluate effectiveness of therapeutics in models that represent the cell types affected in the disease are encouraged.

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