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Invited Review

INVITED REVIEW

Proteostasis disturbance in amyotrophic lateral sclerosis

Danilo B. Medinas[1,2,3,*], Vicente Valenzuela[1,2,3] and Claudio Hetz[1,2,3,4,5,*]

[1]Biomedical Neuroscience Institute, Faculty of Medicine, University of Chile, Santiago, Chile, [2]Program of Cellular and Molecular Biology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile, [3]Center for Geroscience, Brain Health and Metabolism, Santiago, Chile, [4]Buck Institute for Research on Aging, Novato, CA, USA and [5]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, USA

*To whom correspondence should be addressed at: Institute of Biomedical Sciences (Building B, Second floor), University of Chile, Independencia 1027, PO BOX 70086, Santiago, Chile. Tel: +56 229786506; Email: dmedinas@med.uchile.cl (D.B.M.); chetz@med.uchile.cl and chetz@hsph.harvard.edu (C.H.)

Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting motoneurons in the brain and spinal cord leading to paralysis and death. Although the etiology of ALS remains poorly understood, abnormal protein aggregation and altered proteostasis are common features of sporadic and familial ALS forms. The proteostasis network is decomposed into different modules highly conserved across species and comprehends a collection of mechanisms related to protein synthesis, folding, trafficking, secretion and degradation that is distributed in different compartments inside the cell. Functional studies in various ALS models are revealing a complex scenario where distinct and even opposite effects in disease progression are observed depending on the targeted component of the proteostasis network. Importantly, alteration of the folding capacity of the endoplasmic reticulum (ER) is becoming a common pathological alteration in ALS, representing one of the earliest defects observed in disease models, contributing to denervation and motoneuron dysfunction. Strategies to target-specific components of the proteostasis network using small molecules and gene therapy are under development, and promise interesting avenues for future interventions to delay or stop ALS progression.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal motoneuron disease characterized by muscle weakness, spasticity, atrophy, and paralysis, causing about 1 in 500 adult deaths typically within 3 to 5 years after diagnosis due to respiratory failure (1–3). The pathological hallmark of ALS is the selective degeneration of motoneurons in the spinal ventral horn, most brainstem nuclei and cerebral cortex (1–3). Similar to other neurodegenerative diseases, the majority of ALS cases are sporadic (sALS), while approximately 10% are familial (fALS) and caused by mutations in multiple genes (1,3). Together, expanded G_4C_2 hexanucleotide repeat in the intronic region of C9orf72 and mutations in the genes encoding cytosolic superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), and fused in sarcoma (FUS) account for around 60% of fALS cases (1,4). The recent discovery of C9orf72 mutations as a common genetic cause of fALS and frontotemporal dementia has profoundly changed our understanding of ALS (5,6), where the disease is now considered part of a spectrum of neurological disorders instead of simply a neuromuscular disease (2).

Several mechanisms have been implicated in the pathogenesis of ALS, among them oxidative stress, mitochondria dysfunction, excitotoxicity, defective axonal transport, inflammation, and glia activation (1,4). Although a combination of mechanisms may contribute to the aggressive development

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Box 1. Definition of proteostasis and its components

Autophagy: self-degradative process with functions including the removal of misfolded or aggregated proteins, and also damaged organelles.
Calnexin and calreticulin cycle: One major folding and quality control pathway for glycoproteins in the ER and it is mediated by the lectin chaperones calnexin and calteticulin, which form a complex with the protein disulfide isomerase ERp57, assisting disulfide bond formation.
ER stress: It is the cellular condition involving the accumulation of misfolded/unfolded proteins at the ER. ER stress activates UPR stress sensors to adapt to stress or trigger apoptosis of irreversibly-damaged cells.
ER-associated protein degradation (ERAD): designates a cellular pathway that targets misfolded proteins from the ER to ubiquitination and subsequent degradation in the cytosol by the proteasome.
Integrated stress response (ISR): It is a eukaryotic cell adaptive pathway, which is activated by a range of stress conditions that converge into the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), which leads to a decrease in global protein synthesis and the induction of selected genes that promote recovery of homeostasis or the induction of cell death.
Proteostasis: It is a portmanteau of the words protein and homeostasis. Refers to the concept of integrated biological pathways within cells that control the biogenesis, folding, trafficking and degradation of proteins present within and outside the cell.
Stress granules: Cytosolic structures composed of assembled ribonucleoproteins to stop protein translation under a variety of cellular stresses.
Ubiquitin proteasome pathway (UPS): It is the principal mechanism for protein catabolism in the cell. Degradation of a protein via this pathway involves two discrete and successive steps, tagging or conjugation of the substrate protein by the covalent attachment of multiple ubiquitin molecules; and the subsequent degradation of the tagged protein by the 26 S proteasome.
Unfolded protein response (UPR): A signal transduction pathway that is activated in response to an accumulation of unfolded or misfolded proteins at the ER lumen. The UPR mediates the adaptation to protein-folding stress or the elimination of non-functional cells by apoptosis.

of motor dysfunction in ALS, the primary factors underlying this global disturbance remain poorly understood. Of note, a transversal factor associated with ALS and other neurodegenerative diseases is the propensity of certain mutant proteins to misfold and assemble into neurotoxic oligomers and protein aggregates (7,8). Furthermore, proteinaceous inclusions containing such proteins are also observed in sporadic cases of neurodegenerative disorders as well, implying that disturbances in protein folding and quality control mechanisms may cause wild-type proteins to misfold. For instance, the detection of misfolded forms of SOD1 and intracellular inclusions of TDP-43 in affected motoneurons has been consistently reported in sALS and fALS patient tissue (9–11). In this manner, altered protein homeostasis (referred to as proteostasis (12)) is a common feature of ALS as well as other neurodegenerative diseases (7,13–15).

Proteostasis comprises a network of interconnected quality control processes maintaining the functional proteome (12,16) (Fig. 1). Thus, adaptive mechanisms in the cell enhance the expression of chaperones, foldases, and protein degradation mechanisms under proteotoxic stress (Box 1) (16). Accumulating evidence suggests that perturbation in the eficiency of the protein-folding machinery may account for selective vulnerability of motoneurons in ALS (17,18). In line with this notion, strategies to enhance the clearance of abnormal proteinaceous aggregates afford protection against motoneuron degeneration, extending survival in pre-clinical models of the disease (19,20). However, the exact molecular mechanisms driving the misfolding and aggregation of ALS-linked proteins in most common sporadic forms and its impact to motoneuron homeostasis continue as a subject of intense investigation.

The recent identification of genes associated with proteostasis control as risk factors to develop ALS and many functional studies showing the benefits of targeting proteostasis to alleviate motoneuron degeneration support a major role of the pathway in ALS pathogenesis. Here, we overview different aspects of the proteostasis network that are affected in ALS models and patients, highlighting selected disease genes and possible strategies for future therapeutic interventions (Table 1).

Ubiquitin Proteasome System

The degradation of most intracellular proteins occurs through the ubiquitin proteasome system (UPS), which employs polyubiquitin chains to label proteins for proteolysis and a vast array of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligases (E3) enzymes to assure protein turnover in a tightly regulated spatio-temporal manner (21). The accumulation of ubiquitin-positive inclusion bodies in sALS and fALS, in addition to several disease models, has been linked to UPS dysfunction [reviewed in (22)]. The catalytic activity of the proteasome has been shown to be significantly reduced in sALS tissue (23). Cell culture studies have demonstrated that the ALS-linked mutant SOD1 is a substrate of the UPS, and it is selectively recognized by E3 ubiquitin-ligase like Dorfin (24). Importantly, mutant SOD1 accumulates as polyubiquitinated high-molecular-weight species, suggesting the proteasome fails to cope with the burden misfolded mutant SOD1 species (24,25). In fact, studies in mutant SOD1 transgenic mice indicated that the proteasome activity is already impaired as early as 45 days of age, decreasing substantially as the disease progresses (26–28).

In addition to the extensive literature in mutant SOD1 models, the discovery of ALS genes related to UPS supports its dysfunction as part of the etiology of the disease (29) (Fig. 2). Expression of RAN (Repeat-associated non-ATG translated) peptides of C9orf72 repeat expansion has been associated with proteasome impairment (30). The ATPase valosin-containing protein (VCP)/p97 is a member of the AAA (ATPase associated with diverse cellular activities) family, which employs ATP hydrolysis to structurally remodel client proteins. VCP/p97 is able to directly bind to ubiquitylated substrates or interact with ubiquitin conjugates through adaptor proteins to assist

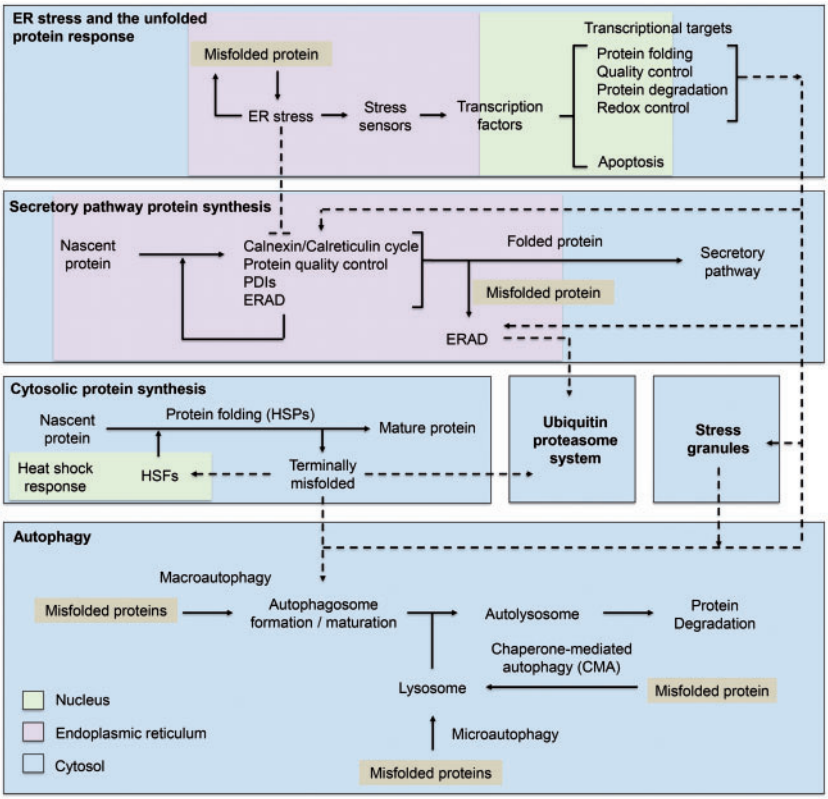

Figure 1. The proteostasis network. Schematic representation of the eukaryotic proteostasis network including the unfolded protein response (UPR), the heat shock response (HSR), the ubiquitin–proteasome system (UPS), autophagy, and stress granules. These protein quality control systems are mechanistically interconnected to promote dynamic adaptation to protein-folding stress. The HSR is activated upon accumulation of misfolded proteins by multiple mechanisms, highlighting the transcriptional regulation by the heat shock factor-1 (HSF1). The UPR is a complex and integrated signal transduction pathway evolved to overcome the accumulation of unfolded or misfolded proteins at the ER lumen or to trigger cell death on irreversible stress. The UPS is the major pathway for non-lysosomal degradation of intracellular proteins whose central event is the covalent linkage of ubiquitin to target proteins that are then recognized by the 26 S proteasome for proteolysis in the cytosol. The autophagy pathway comprises a catabolic process involved in the degradation of protein aggregates and damaged organelles by lysosomes and can be directly activated by the UPR. Finally, stress granules sequester ribonucleoproteins to stop protein translation under a variety of conditions threatening the cell.

proteasomal degradation (31). Mutations in VCP/p97 have been found to cause autosomal dominant ALS, and are also reported in families with inclusion body myopathy, Paget disease, and frontotemporal dementia (32). An ALS-associated VCP/p97 mutant has disrupted interaction with proteasome affecting substrate presentation for proteolysis (33). Another ALS-linked gene associated with UPS encodes for ubiquilin-2, a ubiquitin-like protein involved in delivering cargo to proteasome (34). Of note, ubiquilin-2 mutations are also implicated in X-linked ALS and ALS/dementia (35). Mutations in ubiquilin-2 result in impaired protein degradation with the accumulation of ubiquilin-2-positive skein-like inclusions (35). Transgenic mice overexpressing mutant ubiquilin-2 develop motoneuron disease with muscle denervation and accumulation of ubiquitylated TDP-43 inclusions in the cytoplasm (36). Indeed, TDP-43 has been shown to be a substrate of the UPS (37), and the genetic ablation of proteasome components causes TDP-43 mislocalization and motoneuron pathology (38). Moreover, mutations in cyclin F (CCNF), a component of E3-ubiquitin ligase complex, have been reported in familial and sporadic ALS cases (39). Interestingly, expression of mutant CCNF leads to abnormal accumulation of ubiquitylated proteins including TDP-43 (39). Overall, although a reduced activity of the UPS has been shown to occur in ALS models and human tissue, no functional studies are available to test the significance of these alterations to the progression of the disease. The fact that most ALS-related protein aggregates co-localize with ubiquitin suggests an active role on promoting histopathological changes.

Autophagy

Autophagy is an intracellular lysosome degradation system responsible for the clearance of cytoplasmic components and organelles (40). Macroautophagy, hereafter referred as autophagy, is the most common autophagic pathway involving the formation of double membrane structures called autophagosomes, which engulfs substrates for their transport and delivery to lysosomes (Fig. 2). Other two forms of autophagy are known including chaperone-mediated autophagy (CMA) which mediates the degradation of soluble cytosolic proteins by a direct translocation into the lysosome. Microautophagy is mediated by direct lysosomal engulfment of the cytoplasmic cargo. The sequence of events in autophagy encompasses (i) translocation and initiation, (ii) elongation and recruitment, (iii) completion, and (iv) lysosome fusion and degradation (41). The basal autophagy activity appears to be essential for maintaining proteostasis and viability of neurons as the selective genetic ablation of autophagy-related genes (ATGs) in the CNS causes spontaneous neurodegeneration with the accumulation of typical ubiquitin-positive cytoplasmic inclusion bodies, associated

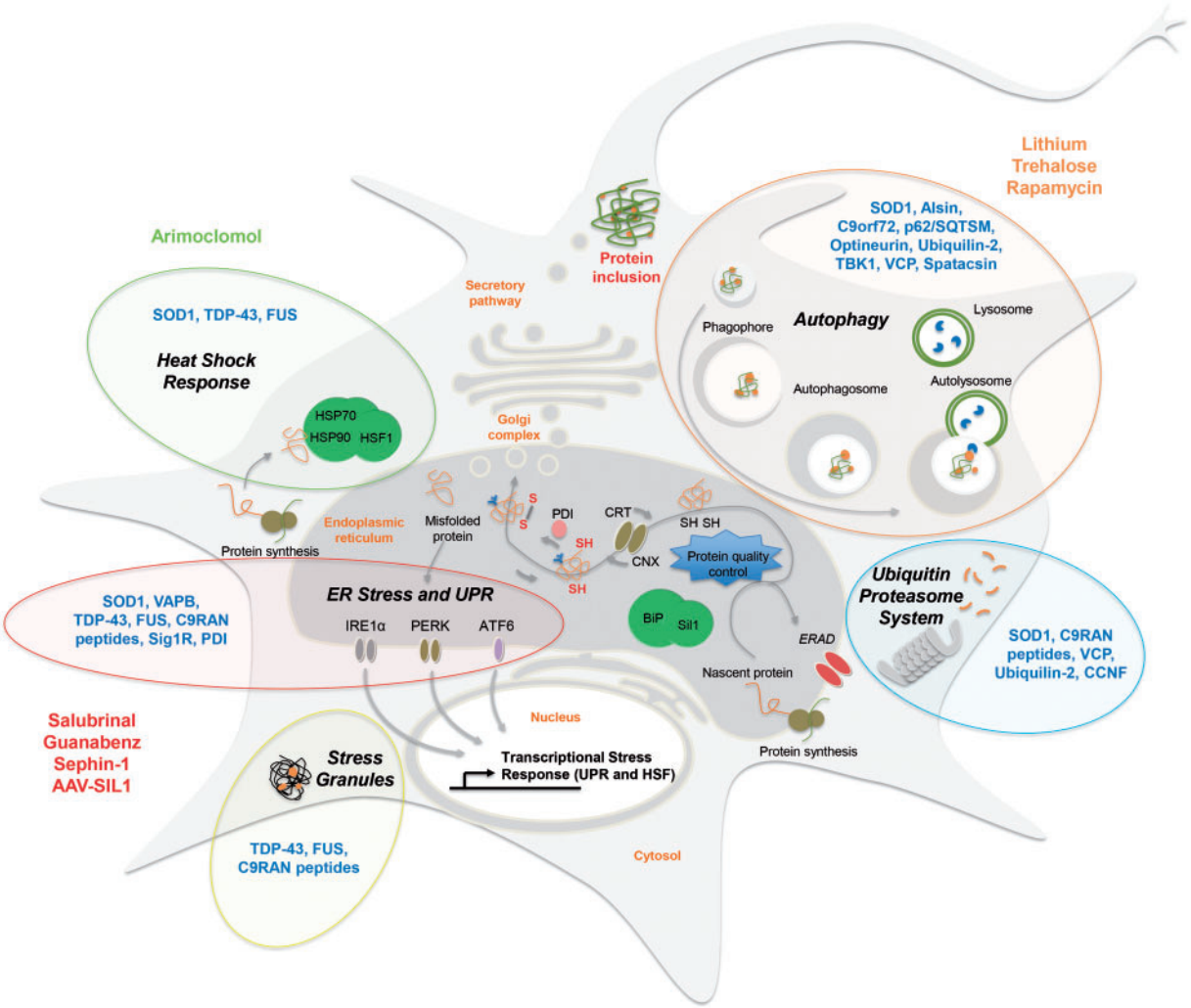

Figure 2. Contribution of ALS genes to proteostasis impairment in ALS. Different ALS-linked mutant proteins negatively impact the proteostasis network. The group of ALS proteins and the prototypic therapeutic tools associated with each proteostatic module are indicated. Misfolding and aggregation-prone proteins such as SOD1, TDP-43, FUS, and C9-RAN peptides can overwhelm the degradative capacity of motoneurons leading to global catabolic failure (UPS and autophagy), in addition to interacting with specific components of the proteostasis network leading to their dysfunction (*e.g.* inhibition of ERAD by mutant SOD1 and stress granules dynamics by mutant TDP-43 and FUS). On the other hand, mostly loss-of-function mutations in ALS proteins cause deficiency of proteostasis pathways (*e.g.* impaired UPR signaling by mutant VAPB), causing damage to organelles and accumulation of misfolded proteins that fuel deleterious vicious cycles precipitating motoneuron degeneration with age.

with neuronal loss, motor impairment and early death (42,43). The autophagy pathway has been proposed as an efficient degradation system to clear out most aggregate-prone proteins linked to ALS including TDP-43 and SOD1 (44,45). Interestingly, in addition to be recognized as cargoes to the autophagy pathway, ALS-linked genes have been shown to impair autophagy activity (Fig. 2), possibly enhancing the accumulation of protein aggregates and damaged organelles (46).

The initiation of the autophagy process is mediated in part by a protein kinase complex that responds to upstream signals (Atg1 and Atg13 in yeast). The serine/threonine protein kinase mTOR (mammalian target of rapamycin) suppresses the pathway under nutrient-rich conditions (40). mTOR-independent autophagy pathways have also been reported that are triggered by changes on calcium and inositol signaling (47). The nucleation and formation of autophagosome are regulated by protein complexes involving the generation of phosphatidylinositol-3-phosphate (PI_3P), including the class III phosphatidylinositol-3-kinase (PI_3K) Vps34, which mediates the localization of other autophagy-regulatory proteins to the pre-autophagosomal structure (41). The nucleation complex is also composed by Beclin 1 (BCL-2-interacting protein). Beclin-1 is negatively regulated through the interaction with BCL-2 and BCL-X_L at the ER membrane (48). Vesicle expansion is carried out by the covalent conjugation of ATG12 to ATG5, which in association with ATG16L1, translocates to the membrane of early autophagosomes and promotes the conjugation of microtubule-associated protein light chain 3 (LC3) to phosphatidylethanolamine (PE) (40). Upon conjugation, the soluble LC3-I translocates to the autophagosome membrane where it is then referred to as LC3-II (49). Finally, autophagosomes fuse with acidic lysosomes to acquire hydrolytic activity, forming the autophagolysosome where cargo is degraded (40). The delivery of protein aggregates to autophagosomes is in part mediated by the adapter protein p62/SQSTM (50). An enhancement in autophagy levels has been reported in ALS patients and mouse models (51–55), observing alterations in different steps of this pathway (Fig. 2) that are presented here according to the

Table 1. Functional studies of proteostasis pathways in ALS models

Approach/Process	Model/Treatment	Mechanism of Action	Effect on ALS Features	Ref
Genetic manipulation				
ER stress/UPR	PERK haplo-insufficiency	Decreased eIF2α phosphorylation	Disease exacerbation in mSOD1 model, enhanced SOD1 aggregation	Wang *et al.*, 2011
	GADD34 haplo-insufficiency	Sustained translation repression	Decreased mSOD1 aggregation and extended lifespan in mSOD1 model	Wang *et al.*, 2014
	ATF4 KO	Decreased apoptosis	Extended lifespan in mSOD1 model	Matus *et al.*, 2013
	XBP1 cKO	Impaired ERAD, increased autophagy	Decreased SOD1 aggregation, neuroprotection, and extended lifespan in mSOD1 model	Hetz *et al.*, 2009
Autophagy	Beclin1 haplo-insufficiency	Decreases autophagy levels	Extended lifespan in mSOD1 model	Nassif *et al.*, 2014
			Reduced lifespan, exacerbated disease in mSOD1 model	Tokuda *et al.*, 2016
UPS	Rpt3 cKO	Deficiency of proteasome subunit	Locomotor dysfunction in wild-type mice	Tashiro *et al.*, 2012
HSPs	HSP70 OE	Increased chaperone activity	No effect on disease progression in mSOD1 model	Liu *et al.*, 2005
	HSP27 OE	Increased chaperone activity	Slight changes in disease progression in mSOD1 model. No changes in survival. Motoneuron protection	Sharp *et al.*, 2008
			No changes in survival in mSOD1 model. No protection in motoneurons	Krishnan *et al.*, 2008
	BAG1 OE	Increased chaperone activity	No effect on disease progression in mSOD1 model	Rohde *et al.*, 2008
	αB-crys OE	Increased chaperone activity	No therapeutic benefit in mSOD1 model	Xu *et al.*, 2015
	HSP110 OE	Increased chaperone activity	Extended survival in mSOD1 mice	Nagy *et al.*, 2016
	AAV-SIL1	Co-chaperone activity	Decreased misfolded mSOD1 and muscle denervation.	Filézac de L'Etang *et al.*, 2015
Pharmacological approach				
ER stress/UPR	Salubrinal	Sustained translation repression by inhibition of GADD34/protein phosphatase 1 (PP1) and CReP-PP1C	Increased survival of mSOD1 model and enhanced formation of stress granules in mutant TDP-43 mice	Saxena *et al.*, 2009 Walker *et al.*, 2013
	Guanabenz	Sustained translation repression by inhibition of GADD34/PP1 complex	Increased survival of mSOD1 model	Wang *et al.*, 2014 Jiang *et al.*, 2014
			Decreased survival of mSOD1 model	Vieira *et al.*, 2015
	Sephin-1	Sustained translation repression by inhibition of phosphatase regulatory subunit PPP1R15A	Increased survival of mSOD1 mice	Das *et al.*, 2015
Autophagy	Rapamycin	Inhibitor of mTORC1	Decreased lifespan in mSOD1 model	Staats *et al.*, 2013
			Increased survival in mutant TDP-43 mice	Wang *et al.*, 2012
	Trehalose	Glucose transport inhibitor	Reduced SOD1 aggregates, increased survival of mSOD1 model	Castillo *et al.*, 2013 Zhang *et al.*, 2014
	Lithium	Inhibitor of inositol-monophosphatase 1	Increased survival of mSOD1 mice	Fornai *et al.*, 2008
	Spermidine, carbamazepine, and tamoxifen	Enhanced autophagy	Rescue motor function in mutant TDP-43 mice	Wang *et al.*, 2012
HSPs	Arimoclomol	Inducer of heat shock response	Reduced mSOD1 aggregates, increased survival	Kieran *et al.*, 2004

AAV: adeno associated vectors; cKO: conditional knockout; mSOD1: mutant SOD1; OE: over expression

sequential flux of cargoes from autophagosome formation to lysosome-mediated degradation.

Initiation

The core of the autophagosome membrane nucleation complex is formed by three proteins, the class III PI_3K Vps34, Vps15 and Beclin 1. Alsin is a GDP/GTP exchange factor for the small GTPase Rab5 (56), which acts at an early stage of autophagosome formation in complex with Vps34/Beclin 1 (57). Loss-of-function mutations in Alsin cause recessive form of ALS (58,59). Targeting alsin in a mutant SOD1 model led to increased accumulation of protein aggregates and autophagosome-like vesicles in addition to p62/SQSTM and LC3-II, indicating its participation in autophagy initiation in ALS (60). The hexanucleotide repeat expansion in C9orf72 gene may cause toxicity due to haploinsufficiency [reviewed in (61)]. Interestingly, C9orf72 has been shown to regulate

autophagy at different levels. Acting as a Rab1a effector, C9orf72 controls the Rab1a-dependent trafficking of the ULK1 autophagy initiation complex to the phagophore (62). C9orf72 also contributes to autophagosome maturation (63,64). The loss of C9orf72 causes autophagy dysfunction with the accumulation of p62/SQTSM aggregates (62–64), sensitizing neurons to proteotoxic stress (63,64). Thus, the production of C9 RAN-peptides aggregates toghether with a deficiency in the autophagy pathway could work synergistically to promote motoneuron demise. *In vitro* studies indicated that mutant SOD1 abnormally interacts with the Beclin-1/BCL-X_L complex, inducing the over activation of autophagy (65). Genetic targeting of Beclin-1 expression using haplo-insufficient mice delayed disease progression in mutant SOD1 transgenic animals possibly due to a normalization in autophagy levels (65). However, in another study, deletion of one copy of Beclin-1 enhanced SOD1 aggregation and disease severity (66).

The pharmacological stimulation of autophagy in ALS revealed a complex scenario where the outcome on disease progression depends on the ALS model used and whether mTOR-dependent or -independent pathways are targeted (55,67–70). Rapamycin has been classically used to enhance autophagy by inhibiting mTOR (71), producing positive effects when administered to several models of neurodegeneration (72–75). However, treatment of ALS models with rapamycin has provided conflicting results. Administration of rapamycin to mutant SOD1 mice to exacerbated the disease possibly due to enhanced apoptosis (55), consistent with the idea that the pathway is over activated in the model. Other studies showed no effects of rapamycin treatment (76,67). Intriguingly, activation of mTOR has been shown to mediate neuroprotection in mutant SOD1 mice by mild stimulation of motoneurons excitability (77). In contrast, mTOR inhibition with rapamycin protected mutant TDP-43 mice to undergo neurodegeneration (68). Similarly, mTOR-independent inducers of autophagy protected mutant TDP-43 mice (68). Interestingly, trehalose, a disaccharide that engages mTOR-independent autophagy (78,79), protected mutant SOD1 against degeneration (69,70). The administration of lithium has also been shown to enhance formation of autophagic vacuoles and afford neuroprotection in ALS mice (80). However, these studies are controversial.

Substrate delivery

Autophagy cargo is loaded to autophagosome by specific receptors employing ubiquitin-associated (UBA) and LC3-interacting region (LIR) domains to bring ubiquitylated substrates into LC3-II vesicles. Mutations in the autophagy receptors p62/SQTSM, optineurin, and ubiquilin-2 have been linked to ALS (35,81–83), possibly impairing the delivery of substrates to the autophagosome. Mutations in p62/SQTSM map to the LIR domain and can prevent the interaction with LC3-II (84). Along the same line, optineurin has been demonstrated to participate in Parkin-dependent mitophagy through recognition of ubiquitylated proteins on mitochondria surface followed by the association with LC3-II membranes and engulfment of the damaged organelle by the autophagosome, a process disrupted by the ALS-linked mutation in optineurin (85). Apart from its function in the UPS, ubiquilin-2 has also been described as an autophagy mediator by promoting the recruitment of ubiquitylated substrates to autophagosomes (86). Interestingly, ubiquilin-2 co-localizes with optineurin in p62/SQTSM and ULK1 vesicles during autophagy initiation and ALS mutant loses this property possibly leading to autophagy defects (87). Finally, loss-of-function mutations in TANK-binding kinase 1 (TBK1) have been linked to ALS (88,89). TBK1 is known to phosphorylate p62/SQTSM and optineurin (89). Thus, several ALS mutations may impact the load of cargoes to autophagosomes compromising autophagy flux.

Maturation

As presented before, VCP participates in the UPS and ALS mutations impair delivery of substrates to the proteasome (33). In addition, VCP mutations also appears to affect autophagosome maturation, causing accumulation of ubiquitin and p62/SQTSM-positive aggregates and cytosolic TDP-43 (90,91). Of note, a genetic screen for modifiers of stress granules (see Box 1) has uncovered a novel function of VCP in controlling the degradation of these membrane-less organelles through autophagy (92). This study shows that VCP deletion or mutations lead to impaired clearance of stress granules (92).

Degradation

Autophagy culminates with the fusion of autophagosomes to lysosomes forming autolysosomes for the digestion of cargo. Mutations in Spatacsin, a protein that participates in autophagic lysosome biogenesis (93), cause autosomal recessive hereditary spastic paraplegia (HSP) and autosomal recessive juvenile ALS (94). Disruption of Spatacsin in mice leads to neurodegeneration associated with accumulation of autofluorescent material that co-localizes with lamp1 and p62/SQTSM, revealing defective autolysosome turnover (95). Overall, these data suggest a complex concept where therapeutics to target autophagy in ALS should aim not only to enhance the activity of the pathway, but also to restore the specific autophagy defects observed in the disease to avoid the accumulation of damaged organelles and other cargoes.

Stress Granules

Stress granules are the assemblies of ribonucleoprotein particles (RNP) stalled in translation initiation that are induced by various forms of cellular stress (96). These are highly dynamic structures composed of core particles and a more fluid shell that can rapidly exchange content with the cytosol, relying on an extensive network of protein–protein interactions and post-translational modifications that ultimately determine stress granule properties in a context-dependent manner (96). When stress granules disassemble, the RNP can return to translation or be targeted for degradation by autophagy (96). The discovery of mutations in TDP-43 and FUS has drawn much attention to altered RNA metabolism in ALS (1,97–102). The aberrant formation of stress granules consists a mechanistic link between proteostasis disturbance and aberrant RNA metabolism (1,103). The biophysical driver for assembly of these membraneless organelles consists of liquid–liquid phase separation mediated by the low-complexity domains (LCD) of RNA-binding proteins such as TDP-43 and FUS (104). Interestingly, ALS mutations in TDP-43 and FUS tend to cluster in the LCD conferring a more rigid structure to stress granules that could impair their clearance by autophagy and seed formation of the classical intracytoplasmic inclusions (1,105). The arginine-containing C9orf72 RAN-peptides were recently shown to interact with LCD of RNA-binding proteins, affecting phase separation and disturbing the dynamics and function of stress granules and other membraneless structures (106). Overall, ALS-related proteins may not only accumulate

into stress granules, but also alter the dynamics of their assembly-disassembly (Fig. 2) (107).

Cytosolic Chaperones

The proper folding of proteins in the crowded intracellular milieu depends on concerted action of chaperones, highlighting the members of heat shock proteins (HSP) that assist not only protein folding or re-folding, but also recognize terminally misfolded proteins and aggregates targeting them to degradation (108). The heat shock response encompasses a vast array of proteins including chaperones, chaperonins, co-chaperones, nucleotide exchange factors, among others that are induced by heat shock factors (HSF) during cellular stress and have been linked to numerous pathological states, including neurodegenerative diseases (109). There is well-documented association of HSP members with mutant proteins and inclusions of ALS models and patient tissue (Fig. 2) (110–112). It has been argued that abnormal interaction of mutant ALS proteins with HSP members may lead to toxicity due to reduced chaperoning activity in affected tissue (113). Numerous studies have shown that enhancing HSP expression reduces SOD1 aggregation and toxicity in cell culture (114–116), whereas overexpression of HSP in transgenic mouse models led to divergent results (117–122). This complex scenario may arise from intrinsic differences of the mutant proteins, their aggregated species, and subcellular location (123). The engineering of HSP has proven efficient to prevent misfolding and promote disaggregation of TDP-43 and FUS (124), consisting an interesting area for further translational efforts (125). In addition, enhancing HSF activity and expression may also offer therapeutic opportunities to treat motoneuron degeneration (126,127). Finally, the pharmacological induction of HSP has been proven protective in ALS transgenic mice (128,129), and is currently under evaluation in a Phase II/III trial for treatment of SOD1 FALS patients (130). Compelling evidence linking cytosolic chaperones to ALS at the functional level *in vivo* is still largely missing. Importantly, strategies to use small molecules with "chemical chaperone" activity such as TUDCA and 4-PBA have been shown to protect against experimental ALS based on mutant SOD1 expression (18).

ER Proteostasis

Several unbiased studies in transgenic SOD1 mouse models, induced pluripotent stem cell (iPSC)-derived patient motoneurons, and post-mortem tissue have identified ER stress as an early and transversal pathogenic mechanism associated with selective vulnerability of motoneurons in ALS (131–138). ER stress is caused by abnormal levels of misfolded proteins in the ER lumen, engaging a signal transduction pathway termed unfolded protein response (UPR). The UPR mediates initial adaptive responses to restore proteostasis through various mechanisms including inhibition of protein translation mediated by the phosphorylation of the eukaryotic initiation factor 2α (eIF2α), in addition to transcriptional induction of chaperones, foldases, protein quality control and degradation systems, lipid biosynthesis, among others (139). Under chronic ER stress, the UPR shifts its signaling toward a terminal phase to eliminate irreversibly damaged cells through apoptosis (140). Under pathological conditions of chronic ER stress, as observed in numerous neurodegenerative diseases (13–15), the terminal UPR engages pro-inflammatory and apoptotic cascades leading to cell death (141,142).

The mammalian UPR is mediated by the activation of three stress transducers named activating transcription factor 6 (ATF6) alpha and beta, protein kinase R (PKR)-like ER kinase (PERK), and inositol-requiring enzyme 1 (IRE1) alpha and beta to communicate protein-folding status from the ER lumen to cytosol and nucleus (Figs 1 and 2) (16). Upon ER stress, membrane-bound ATF6 translocates to the Golgi apparatus and is cleaved by site-1 and site-2 proteases to release the soluble transcription factor ATF6f into the cytosol (143). PERK activation leads to the direct phosphorylation of eIF2α resulting in attenuated translation and induction of the transcription factor ATF4, which regulates folding, autophagy, amino acid and redox metabolism (144). Under sustained ER stress, ATF4 promotes the expression of the pro-apoptotic transcription factor CHOP (145). CHOP also induces GADD34, a component of the phosphatase complex that dephosphorylates eIF2α and restores normal protein synthesis (146). Several other stress kinases, including PKR and GCN2, phosphorylate eIF2α, a pathway known as the integrated stress response (IRS) (147). IRE1 is a kinase and endoribonuclease that upon ER stress catalyzes the unconventional splicing of X-box-binding protein 1 (XBP1) mRNA, leading to the production of a potent transcription factor termed XBP1s (148). During the adaptive UPR, XBP1s induces the expression of ER chaperones and co-factors, ER-associated protein degradation (ERAD) components, and lipid biosynthesis to increase ER network and protein folding and quality control capacity (140). When ER stress is chronic, IRE1 is overactivated through assembly into high-order oligomers and reduces its substrate specificity to catalyze degradation of mRNA and microRNAs, an activity termed regulated IRE1-dependent decay (RIDD) (149). Furthermore, IRE1 can interact with cytosolic components, including adapter proteins, to fine-tune UPR outputs in a dynamic fashion, comprising a protein platform termed 'UPRosome' (150). For instance, IRE1 can be coupled to JNK and NF-κB pathways through the adaptor protein TRAF2 to induce apoptosis upon prolonged ER stress (151,152).

UPR signaling

The ALS-linked mutant SOD1 has been shown to interact and inhibit Derlin-1, a component of the ERAD system (153). ERAD dysfunction leads to chronic ER stress and motoneuron death through the IRE1-ASK1 pathway (153), which has recently also been shown to involve homeodomain-interacting protein kinase 2 (HIPK2) leading to the activation of JNK (154). Interestingly, activation of HIPK2 positively correlates with TDP-43 proteinopathy in familial C9orf72 and sporadic ALS cases, supporting a broad role of ER stress in ALS pathogenesis (154). We have performed genetic manipulation to assess the functional impact of the UPR in ALS models by knocking-out XBP1 or ATF4 (51,155). Genetic ablation of ATF4 increased the lifespan of mutant SOD1 mice possibly due to decreased expression of the apoptotic factors CHOP and BIM (155). On the other hand, PERK haplo-insufficiency hastens disease onset and progression associated with reduced translational repression, and enhanced the accumulation of mutant SOD1 aggregates (156). Boosting the IRS using drugs that maintain eIF2α phosphorylation such as salubrinal, guanabenz or sephin-1, or through genetic ablation of eIF2α phosphatases yields neuroprotection in ALS models with attenuated ER stress (137,157–160). The conditional deletion of XBP1 in the nervous system delayed disease onset and extended lifespan of mutant SOD1 mice. This unexpected observation was explained by a homeostatic balance

between the UPR and the autophagy pathway (51). We found that XBP1 deficiency in mutant SOD1 mice leads to up-regulation of the autophagy pathway, thus boosting degradation of toxic SOD1 aggregates and slowing disease progression (51).

Mutations in vesicle-associated membrane protein-associated protein B (VAPB), an ER protein that has been linked to direct alterations of the activity of ATF6 and XBP1s (161,162), cause familial ALS (163). Most likely, VAPB mutations confer susceptibility of motoneurons to ER stress by perturbing the adaptive capacity of the cell (164).

ER chaperones and quality control pathways

The comparative gene expression profiling of vulnerable and resistant motoneurons in mutant SOD1 mice identified ER stress as the earliest pathological event occurring before any denervation is detected (137). Furthermore, ribosome profiling of motoneurons and glia *in vivo* indicated that ER stress is a major pathological signature of motoneurons, and may mediate cell-autonomous neurodegeneration cascades in mutant SOD1 models (165). The ER orchestrates protein folding and post-translational modifications using an array of chaperones and quality control mechanisms, such as binding immunoglobulin protein (BiP), involved in maintaining stability of newly synthesized proteins translocating into the ER and also the retro-translocation of misfolded proteins to the cytosol through ERAD for subsequent degradation by the proteasome, protein disulfide isomerases (PDI), oxidoreductases that mediate disulfide bond formation in the secretory pathway, and calnexin/calreticulin, two lectin-like chaperones that recognize glycosylated substrates, coupling them with other chaperones and preventing misfolded proteins from trafficking from the ER to Golgi apparatus (Fig. 2).

Altered ER chaperone network has been proposed to underlie differential susceptibility of motoneurons in ALS (18). For instance, the BiP co-chaperone SIL1 was found enriched in resistant while progressively reduced in vulnerable motoneurons over disease course, and SIL1 overexpression using adeno-associated virus was proven to be neuroprotective (138). Genetic findings also support the concept that motoneurons are selectively vulnerable to perturbations to ER function. Mutations in sigma-1 R, an ER chaperone that controls calcium signaling in mitochondria-associated membrane through inositol 1,4,5-trisphosphate (IP_3) receptors (166), cause juvenile form of ALS due to calcium imbalance and ER stress (167,168). Recently, we have identified point mutations in two PDI family members, ERp57 and PDIA1 using targeted sequencing of ALS cases (169). Functional studies indicated that perturbation in the activity of these foldases causes impairment of neuromuscular junction structure and function, which was linked to altered synthesis of synaptic proteins and the development of early alterations in ALS (170). Interestingly, proteomic studies have found ERp57 and PDIA1 as top hits up-regulated in SOD1 rodent models and ERp57 was shown to be the best biomarker for disease progression in patient blood (131,171). Moreover, PDIA1 has been demonstrated to be present in cerebrospinal fluid of sporadic patients and to co-localize with aggregates of ALS proteins (131,132,172,173). Finally, deficiency of calreticulin, which presents substrates for disulfide bond formation by ERp57, led to exacerbated muscle weakness and denervation in the mutant SOD1 mouse model (174). Together, these studies suggested that disrupted ER proteostasis underlays early ALS stages that result in muscle denervation and motor disease onset (Fig. 2).

Conclusion

Significant advances encompassing genetic, cell biology, and histopathological studies suggest that perturbations to the proteostasis network may contribute to early events in ALS pathogenesis, driving neuromuscular dysfunction. The decline of proteostasis buffering capacity with age combined with genetic risk factors adversely affecting distinct nodes of the proteostasis network may operate in a vicious feed-forward cycle. This abnormal process may enhance the accumulation of specific misfolded proteins and, at the same time, may account for the variability in clinical manifestation of ALS that is nowadays understood as part of a disease spectrum including frontotemporal dementia. The interdependence of the proteostasis pathways impaired in ALS may ultimately define the stress threshold to undergo motoneuron degeneration (Fig. 2). The discovery of ALS genes impacting proteostasis notwithstanding, paramount questions regarding the involvement protein misfolding and aggregation in disease etiology need to be addressed: What is the exact nature of toxic protein species? How do these proteins affect neuronal subpopulations? What is the damage threshold leading to proteostasis failure in motoneurons? When does proteostasis decline affect motoneuron functioning during disease evolution? What is the limit for restoring proteostasis and preventing motoneuron loss? Overall, the adaptive capacity of the proteostasis network has been proposed to operate as a protective factor to manage protein folding stress observed in ALS motoneurons, but it may turn into a deleterious pathway when exacerbated responses take place under chronic stress fueling neurodegeneration. Hence, alterations in ER folding components, the autophagy pathway, the UPR and quality control mechanisms arise as fundamental elements in ALS pathogenesis that may hopefully lead to the development of efficacious treatments.

Conflict of Interest statement. None declared.

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