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COMPREHENSIVE INVITED REVIEW

Amyotrophic Lateral Sclerosis Pathogenesis: A Journey Through the Secretory Pathway

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Abstract

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motoneuron degenerative disease characterized by the selective loss of motoneurons in the spinal ventral horn, most brainstem nuclei, and the cerebral cortex. Although approximately 90% of ALS cases are sporadic (sALS), analyses of familial ALS (fALS)-causative genes have generated relevant insight into molecular events involved in the pathology. Here we overview an emerging concept indicating the occurrence of *secretory pathway stress* in the disease process. These alterations include a failure in the protein folding machinery at the endoplasmic reticulum (ER), engagement of the unfolded protein response (UPR), modifications of the Golgi apparatus network, impaired vesicular trafficking, inhibition of protein quality control mechanisms, oxidative damage to ER proteins, and sustained activation of degradative pathways such as autophagy. A common feature predicted for most of these alterations is abnormal protein homeostasis associated with the accumulation of misfolded proteins at the ER, possibly leading to chronic ER stress and neuronal dysfunction. Signs of ER stress are observed even during presymptomatic stages in fALS mouse models, and pharmacological strategies to alleviate protein misfolding slow disease progression. Because the secretory pathway stress occurs in both sALS and several forms of fALS, it may offer a unique common target for possible therapeutic strategies to treat this devastating disease. *Antioxid. Redox Signal.* 13, 1955–1989.

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I. Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is the most common adult-onset motoneuron neurodegenerative disease characterized by muscle weakness, atrophy, and paralysis. Premature death is often observed as a result of respiratory muscle failure. The pathological hallmark of ALS is the selective degeneration of upper and lower motoneurons projecting from the spinal cord, brainstem, and cortex (268). The average disease onset occurs around age 50, with an incidence estimated of 1–2 cases per 100,000 individuals, where men are more frequently affected than women (243). Approximately 90% of ALS cases are referred to as sporadic (sALS), lacking a clear genetic component. Ten percent of the remaining cases are familial (fALS) and many disease-causative genes have been identified by linkage analysis and positional cloning (examples in 35, 63, 94, 136, 160, 243, 327). Those genes include: *Als1*, *Als2*, *Vapb*, *Chmp2b, Dctn1, Tardbp, Fus,* and *Fig4* which encode for superoxide dismutase-1 (ALS1/SOD1), alsin (ALS2), VAMP-associated protein B (VAPB), charged multivesicular body protein 2 B (CHMP2B), dynactin (DCTN1), TAR DNA-binding protein 43 (TDP-43), fused in sarcoma protein (FUS), and lipid phosphatase (FIG4), respectively (Table 1).

There is currently no primary therapy for this disorder, and the only available drug for treatment is an antiglutamatergic compound, riluzole, which has a marginal effect on improving symptoms and only extends lifespan for a few months (14). Several pharmacological agents, including caspase inhibitor zVAD-fmk, creatine, minocycline, and celecoxib have been tested in animal models of ALS, showing promising effects, but with no effects on human patient trials (42, 92, 267). Other drugs such as arimoclomol (43) or talampanel (240) are currently being tested in clinical trials as potential therapy for ALS (5). An area of active therapeutic research is focused on fALS cases caused by dominant mutation in the gene encoding SOD1. Experimental strategies to knockdown mutant *sod1* expression have been already tested in fALS mouse models. Viral-mediated delivery of interfering RNA targeting *sod1* mutant mRNA or direct infusion of antisense oligonucleotide into the nervous system showed surprising therapeutic effects, slowing disease progression and onset, and extending mouse survival (201, 252, 255, 300). Although these results are promising, optimizing gene delivery and addressing safety issues related to viral-mediated gene therapy remains an essential challenge to move the strategy forward and apply it to human patients.

The primary mechanism contributing to progressive motoneuron loss observed in both sALS and fALS remains controversial. Multiple perturbations of cellular function/processes have been uncovered in ALS affected motoneurons. These alterations include impaired energy metabolism, oxidative stress, mitochondrial dysfunction, abnormal calcium homeostasis, cytoskeletal disturbances, altered axonal transport, protein misfolding, accumulation of ubiquitinated protein inclusions, among other events (see examples in 20, 37, 68, 187, 193, 254, 310). In addition, neuroinflammatory and neurotoxic responses related to activation of astrocytes and microglia are also proposed as relevant factors contributing to ALS pathogenesis, supporting the nonautonomous motoneuron cell death hypothesis (see examples in 10, 30, 181, 215, 337). In this article, we specifically focus on relevant reports addressing the role of protein misfolding and secretory pathway stress to ALS. Specialized reviews on the role of neuroinflammation and the participation of non-motoneuron cells in ALS can be found elsewhere (10, 20, 113).

Since sporadic and familial cases of ALS are clinically similar, progress in elucidating the mechanisms underlying different fALS forms may provide insight for an efficient treatment for both disease forms. The contribution of mitochondrial dysfunction in the pathogenesis of ALS had received special attention in ALS research in the last decades and has been extensively reviewed elsewhere (8, 67, 114). The impact of secretory pathway stress is recently becoming a new important aspect of ALS pathogenesis (284). In this review, we aim to give a global view and discuss converging evidence supporting a role of disturbances on protein homoeostasis (hereon referred to as *proteostasis)* networks and organelle stress in fALS and sALS by illustrating a journey through different steps of the secretory pathway. We will start this trip with a first stop in the ER compartment, summarizing its basic functions in protein folding, and then discuss evidence suggesting an involvement of ER stress in ALS pathogenesis. The following stops include a description of alterations reported in the Golgi apparatus (GA) network, axonal/vesicle transport machinery, and the involvement of degradative lysosomal compartments in ALS. In addition, we provide some hints suggesting a crosstalk between secretory pathway stress and the mitochondria through certain forms of oxidative stress and how this could relate to abnormal protein folding.

Table 1. Main Genes Linked to Familial ALS

Gene	*Protein*	*Affected compartment*	*Effects*
als1	Superoxide dismutase-1 (ALS1/SOD1)	ER GA Autophagy Vesicle traffic	UPR activation Interactions with ER-chaperones Induction of ER stress pro-apoptotic genes GA fragmentation Altered secretion Altered anterograde transport Enhanced autophagy ERAD inhibition ER aggregates Abnormal secretion interaction with chromagranins Abnormal ubiquitinated protein inclusions
als2	Alsin (ALS2)	MVE Vesicles traffic	Altered vesicle traffic
vapb	VAMP-associate protein B (VAPB)	ER GA	Interaction with ATF6 sensor, inhibition of XBP-1s ER aggregates GA dispersion
dctn1	Dynactin (DCTN1)	GA vesicles traffic	GA fragmentation Altered retrograde transport
chmp2b	Charged multivesicular body protein 2 B (CHMP2B)	MVE vesicles traffic	Accumulation of autophagosomes
tardbp	TAR DNA-binding protein 43 (TDP-43)	GA and RNA metabolism	Dysfunction on GA morphology Abnormal ubiquitinated protein inclusions Association with stress granules Enhanced autophagy-mediated degradation
fus	Fused in sarcoma protein (FUS)	RNA metabolism	Abnormal ubiquitinated protein inclusions
fig4	Lipid phosphatase FIG4 (FIG4)	Autophagy	Abnormal autophagy, accumulation of p62

II. First Stop: The Endoplasmic Reticulum

A. Endoplasmic reticulum: Primary functions

The ER is an essential subcellular compartment responsible for protein and lipid synthesis. Folded proteins that pass "quality control" are transported through the secretory pathway to reach their final destination. After translocation to the ER lumen through the translocon complex channel, nascent proteins are assisted in their folding by a complex family of chaperones, foldases and co-factors (see reviews in 69, 144, 256). ER key folding mediators are the glucose regulated protein 78 (GRP78; also known as immunoglobulin binding protein: BiP) and 94 (GRP94), protein disulfide isomerases including PDI and ERp57 (also known GRP58), calnexin, and calreticulin, among many other foldases (101, 218, 235).

ERp57 is part of a large family of oxidoreductases responsible for catalyzing the formation, isomerization, and reduction of disulfide bonds (323). ERp57 is classically described as a glycoprotein-specific disulfide isomerase that has a crucial role in the calnexin cycle of protein quality control. By direct association with calnexin and/or calreticulin, ERp57 is recruited to fold substrates bound to these lectin-like chaperones. It is likely that most glycol-polypeptides are released from calnexin/calreticulin/ERp57 in a native and transport competent state. When folding is not completed in a single round of the calnexin cycle, polypeptides enter into an additional folding cycle consisting of a disulfide rearrangement. Glycopolypeptides released from the calnexin cycle that display major folding defects are recognized by BiP/GRP78 and are translocated to the cytosol for proteasome-mediated degradation by the ER-associated degradation (ERAD) machinery.

B. An emergency exit from ER: The ERAD pathway

ERAD is a mechanism employed by the ER protein quality control system and the calnexin cycle to eliminate misfolded or unassembled proteins generated during the folding process (69). The ERAD machinery consists of chaperones, transmembrane proteins, and ubiquitin-associated enzymes that select, target, and retrotranslocate misfolded proteins to the cytoplasm for degradation by the proteasome system (339). These include the ER degradation-enhancing α-mannosidase-like lectins (EDEMs), a class of mannosidase-like proteins directly involved in the recognition and targeting of unfolded proteins for degradation (287). Three EDEM genes, EDEM-1, -2, and -3, have been identified that may share similar functions in delivering ERAD substrates to the retrotranslocation channel (339). EDEM1 associates with the Der1-like domain (DERLIN) family of proteins (124), which are components of the retrotranslocation channel. Disulfide reductases such as ERdj5 cleave the disulfide bonds of misfolded proteins, accelerating ERAD through a physical interaction with EDEM and BiP. ERAD impairment has been implicated in some neurodegenerative diseases associated to protein misfolding, such as Huntington's, Parkinson's, and ALS (see below) (228, 270, 299).

C. Mild ER stress and the unfolded protein response: Adaptive phase

It is estimated that the total protein concentration within the ER lumen reaches $\sim$ 100 mg/ml, a concentration highly prone to protein aggregation which is avoided by an effective folding quality control system (218, 304). Conditions that interfere with ER function consequently lead to abnormal

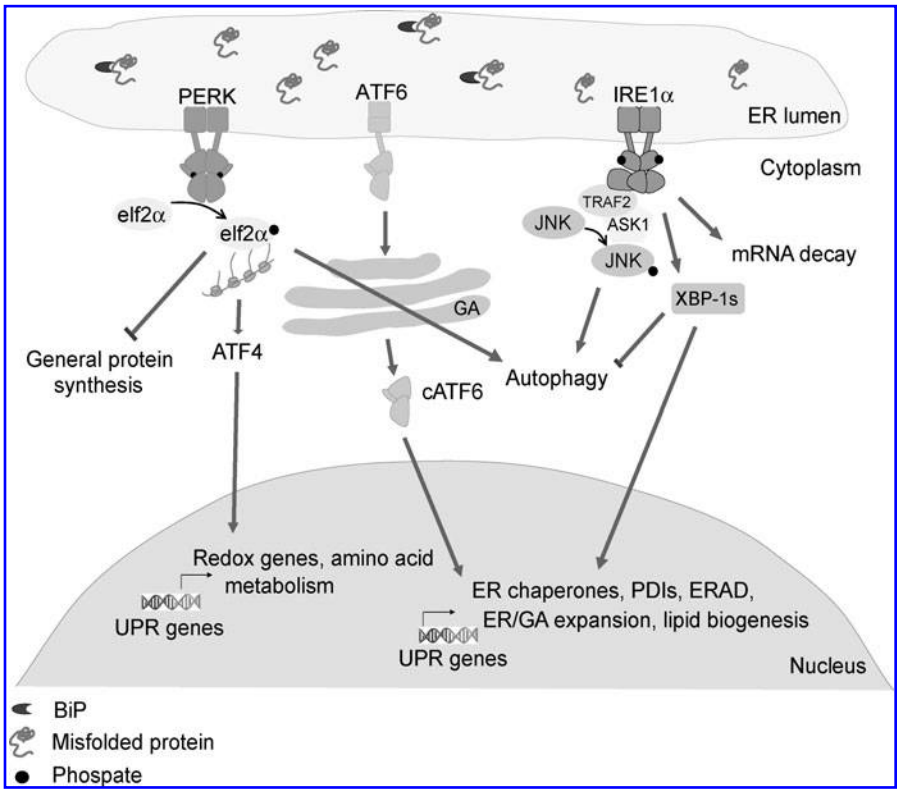

FIG. 1. The unfolded protein response (UPS): adaptive phase. Accumulation of misfolded or unfolded proteins at the endoplasmic reticulum (ER) lumen triggers an adaptive stress response known as the unfolded protein response (UPR). In mammals, there are at least three types of ER stress sensors: IRE1, PERK, and ATF6. In cells undergoing ER stress, IRE1α dimerizes and autophosphorylates, leading to the activation of its endoribonuclease activity at the cytosolic domain. Active IRE1α processes the mRNA encoding XBP1, which is a transcription factor that upregulates many essential UPR genes involved in folding, organelles biogenesis, ERAD, and protein quality control. The RNAse domain of IRE1α also controls the mRNA decay of several ER located proteins. In addition, active IRE1α activates alarm responses such as the ASK1/JNK pathway through the binding of the adaptor protein TRAF2. Alternatively, activation of PERK decreases the general protein synthesis rate through phosphorylation of the initiation factor eIF2α. eIF2α phosphorylation, in contrast, increases the specific translation of the atf4 mRNA, which encodes a transcription factor that induces the expression of genes involved in amino acid metabolism, antioxidant responses, and apoptosis including CHOP/GADD153. A third UPR pathway is initiated by ATF6, a type II ER transmembrane protein encoding a bZIP transcriptional factor on its cytosolic domain and localized at the ER in unstressed cells. Upon ER stress induction, ATF6 is processed at the GA releasing its cytosolic domain, which then translocates to the nucleus increases the expression of some ER chaperones, ERAD-related genes, and protein involved on ER/GA expansion. The IRE1α and PERK pathways also control the induction of adaptive pathways such as autophagy.

protein folding and accumulation of unfolded/misfolded proteins in the lumen, a cellular condition referred to as 'ER stress' (116, 264). ER stress can be originated by diverse cellular alterations, such as abnormal calcium homeostasis, altered redox status, glucose/energy deprivation, expression of mutant genes, and demand for a high secretory activity, among other conditions (287). To alleviate protein folding stress, cells activate an adaptive signal transduction pathway known as the Unfolded Protein Response (UPR) (Fig. 1) (116, 287). Activation of the UPR has diverse cellular consequences, affecting the expression of proteins involved in nearly every aspect of the secretory pathway including genes related to protein entry into the ER, protein folding, ERAD, protein maturation/modification, lipid metabolism, autophagy, and redox metabolism.

UPR signaling transduces information about the protein folding status at the ER lumen to the nucleus by controlling the expression of specific transcription factors through three distinct ER-located stress sensors. These sensors include double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) α/β, and inositol requiring kinase 1 (IRE1α/β) (reviewed in 116, 264, 287). Activation of PERK leads to the phosphorylation and inhibition of eukaryotic translation initiation factor 2 (eIF2α), attenuating general protein translation into the ER, thus decreasing unfolded protein load (19, 105). Additionally, eIF2α phosphorylation augments the specific translation of the mRNA encoding Activation of Transcription-4 (ATF4), a UPR transcription factor essential for the upregulation of many UPR-associated genes that function in amino acid metabolism, redox homeostasis, and apoptosis (1, 106, 167). Therefore, a cellular consequence of PERK activation is the control of genes that helps the cell adapt to the pleiotropic consequences of protein misfolding stress (Fig. 1).

Upon activation, ATF6 translocates from the ER membrane to the GA where it is proteolytically processed, releasing the cytosolic domain which translocates to the nucleus acting as a transcription factor that upregulates several ER chaperones and ERAD-related genes (Fig. 1) (48, 110). IRE1α and its downstream target X-Box-binding protein 1 (XBP1), initiate the third and more conserved adaptive response of the UPR. IRE1α is a serine/threonine protein kinase and endoribonuclease that, upon activation, initiates the unconventional splicing of the mRNA encoding the transcription factor XBP1 (28, 170, 361). This mRNA maturation event promotes the translation of a more stable protein, XBP1s (for spliced XBP1), which translocates to the nucleus controlling the upregulation of a subset of UPR-related genes linked to protein quality control, folding, the ERAD system, and ER/GA biogenesis (264). In the

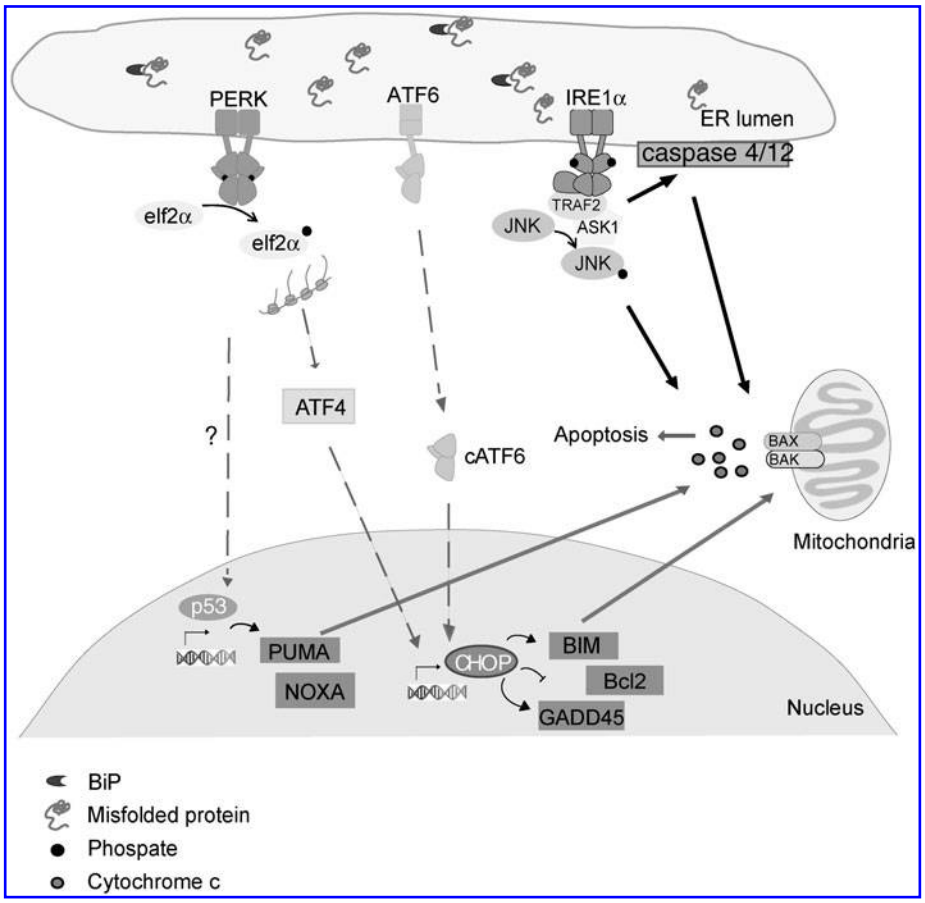

FIG. 2. The unfolded protein response: apoptosis phase. Under chronic ER stress, the UPR induces several redundant mitochondrial independent- and dependent-apoptotic pathways. Activated IRE1α interacts with the adaptor protein TRAF2, leading to the activation of the pro-apoptotic JNK pathway. Activated IRE1α may be also involved in the activation of pro-caspase-12/pro-caspase-4 through interactions with TRAF2. Activated PERK and ATF6 lead to the transcriptional activation of *gadd153* (CHOP) that induces apoptosis possibly by inhibiting expression of the anti-apoptotic gene *bcl-2* and/or through regulation of the pro-apoptotic genes, including *gadd45* and *bim*. PERK/elf2α may control *puma* and *noxa* upregulation through p53. All these events finally promote the release of cytochrome c from the mitochondria controlled by BAX/BAK oligomerization, leading to cell death by apoptosis. Thus, related injuries can initiate transcriptional and cytosolic responses to engage the mitochondrial intrinsic cell death pathway.

transcriptional control of ERAD genes, ATF6 heterodimerizes with XBP-1s to form an active transcription factor. In addition to controlling *xbp1* mRNA processing, the IRE1α RNAse activity degrades a subset of mRNAs encoding certain ER proteins predicted to be difficult to fold (103, 125, 126).

IRE1α has additional functions in cell signaling. The cytosolic domain of activated IRE1α binds to the adaptor protein TNFR-associated factor 2 (TRAF2), triggering the activation of the apoptosis signal-regulating kinase 1 (ASK1) and c-Jun-N terminal kinase (JNK) pathway (151, 228, 330). IRE1α also modulates the activation of the p38, ERK (224) and NF-κB pathways (128), possibly by the binding of the SH2/SH3 containing adaptor proteins Nck and a protein complex between inhibitor κB kinase (IKK)/TRAF2, respectively. However, the function of these UPR signaling branches in the context of protein misfolding is still unclear. The amplitude and kinetics of IRE1α signaling are modulated by the formation of a protein complex where different regulators assemble, a scaffold that we have termed the UPRosome (reviewed in 88a, 116). We and others have identified several components of the UPRosome that can both modulate the activation and attenuation of IRE1α activity (97, 115, 179, 183).

Accumulating reports have provided insight into the potential mechanisms underlying UPR-sensor activation. Under normal conditions, the ER chaperone BiP/GRP78 binds to IRE1α maintaining the protein in an inactive monomeric state (16, 153, 233). In ER stressed cells, BiP preferentially binds to unfolded proteins and thus stops repression of IRE1α, which allows for multimerization and subsequent autophosphorylation, leading to activation of its RNase activity. The structure of the ER luminal domain of yeast and human IRE1α protein has been solved. Evidence in the yeast protein suggested that misfolded proteins at the ER lumen may directly bind to the N-terminal region of IRE1α, facilitating its oligomerization through a binding motif similar to an MHC-like groove (38). *In vitro* binding assays also favor this hypothesis (152). It is not clear if this mechanism also operates in mammalian systems (116, 233), but the regulation of IRE1α dimerization through BiP binding as a sensing mechanism is the most widely accepted model. BiP release and dimerization has been also proposed to be essential for PERK and ATF6 activation (reviewed in 264). In summary, the UPR is an integrated signaling response that orchestrates adaptive processes against ER stress to allow transcriptional reprogramming to maintain proteostasis (Fig. 1).

D. Chronic ER stress and the unfolded protein response: Apoptosis phase

Under chronic ER stress cells undergo cell death by apoptosis (262, 263, 287). Different regulators have been identified, with the BCL-2 family of proteins playing a major role (Fig. 2).The BCL-2 family is composed by both pro- and anti-apoptotic members classified by the presence of at least four conserved BCL-2 homology (BH) domains (57). Intrinsic apoptosis signals converge into the activation of pro-apoptotic "multidomain" members BAX and BAK at the mitochondria, leading to cytochrome c release and apoptosis.

BCL-2 family members that only contain the BH3 domain, termed "BH3-only" proteins, are upstream regulators of BAX and BAK-dependent cell death (24, 150, 362). Two BH3-only proteins, PUMA and NOXA, are strongly induced at the transcriptional level by p53 in cells undergoing prolonged ER stress (Fig. 2) (174, 258). In addition, activation of the BH3-only protein BIM through transcriptional and post-translational activation is essential to induce apoptosis under chronic ER stress responses in cellular and animal studies (155, 208, 250). Thus, direct regulation of the BCL-2 protein family by the UPR provides a direct crosstalk between the ER and mitochondria where protein misfolding-related injuries triggers the activation of the classical intrinsic death pathway at the mitochondria.

Activation of ASK1 and its downstream target JNK regulate apoptosis, at least in part, under irreversible ER stress in an analogous fashion to TNF receptor signaling (139, 195). This model was confirmed by a high-throughput chemical screen searching for inhibitors of ER stress-induced cell death (151). Sustained PERK signaling is also proposed as a pro-apoptotic effecter (177). Expression of ATF4, and possibly ATF6, regulates induction of pro-apoptotic genes such as the CCAAT/enhancer binding protein (C/EBP) homologous (CHOP), also identified as a growth arrest and DNA damage-inducible gene (gadd153) (19). The mechanism by which CHOP leads to cell death is not completely understood. Some proposed mechanisms for the induction of apoptosis by CHOP includes downregulation of BCL-2 expression (196), induction of *bim* transcription (250), and the transcriptional control of *gadd34* (189) (Fig. 1).

In murine cells, the proteolytic processing of the ER-resident pro-caspase-12 has been indirectly associated to the UPR by an interaction with TRAF2 and possibly with active IRE1α (360), but a protein complex between pro-caspase-12/TRAF2/IRE1α has not been reported. Although caspase-12 processing, and its human homologue caspase-4, are well accepted markers of ER stress, their role in apoptosis is still under debate (231, 274). Indeed, the amino acid sequence of caspase-12 clusters with inflammatory caspases and have been shown to participate in inflammatory responses (274, 275). In addition, an alternative caspase, identified as caspase-2, regulates ER stress-mediated apoptosis in different cell types *in vitro* by activating the BH3-only protein BID (56, 329). Many other components of the ER stress apoptosis machinery had been described and the subject is reviewed elsewhere (112, 353).

E. ER alterations in sALS

The etiology of sALS is still unclear. Structural studies have revealed alterations in the structure of subcellular organelles in spinal cord and brainstem motoneurons of sALS patients. Decreased levels of cytoplasmic RNA and rough ER content (chromatolysis), has been described in sALS, suggesting general abnormalities in the protein synthesis system (Table 2) (236). Recent studies from different laboratories suggest that ER stress responses may contribute to both sALS and fALS pathogenesis (2, 121, 129, 149). For example, increased phosphorylation of eIF2α and upregulation of both PDI and BiP/GRP78 have been reported in spinal cord tissue of sALS patients (129, 148), correlating with increased accumulation of ubiquitinated protein inclusions (129). This data was confirmed by a different group that additionally showed increased levels of total PERK, IRE1α and ATF6 together with CHOP, ERp57, and caspase-4 expression in spinal cord tissue derived from sALS cases (3). However, direct evidence for the activation of ATF6, PERK, and IRE1α were not provided in the study. The presence of PDI was reported in cerebrospinal fluid (CSF) from sALS patients, suggesting that UPR-related components could be used as disease biomarkers (3). Our group recently confirmed some of these observations and further demonstrated upregulation of the UPR transcription factors ATF4 and XBP1s in spinal cord tissue from sALS patients (121). We also described the upregulation of EDEM1 in the same samples (121), suggesting that the ERAD pathway is altered in the pathological condition. Additionally, a recent report confirmed the upregulation of CHOP in sALS spinal cord tissue (131). Overall, these correlative reports offer new insight into sALS pathogenesis and suggest a relevant role of protein folding stress in the disease process. Whether ER stress is a cause or a secondary consequence in the pathogenesis of sALS remains to be determined. Nevertheless, recent studies in animal models of fALS have demonstrated a functional role of the UPR in disease initiation and progression (see next sections).

Table 2. Effect Observed at the Secretory Pathway on Sporadic ALS

Compartment/ pathway	*Effects*	*Reference*
ER	Activation UPR components (PERK, IRE1α, (ATF6,XBP-1,ATF4)	Atkin *et al*., 2008 Hetz *et al*., 2009
	Chaperones upregulation (BiP, PDI, GRP58)	Kieran *et al*., 2007 Ilieva *et al*., 2007 Atkin *et al*., 2008 Hetz *et al*., 2009
	PDI nitrosylation	Walker *et al*., 2010
	Apoptotic proteins (CHOP, Caspase-4)	Atkin *et al*., 2008
	Ribosome detachment	Oyanagi *et al*., 2008
	ER dilatation	Oyanagi *et al*., 2008
GA	Fragmentation	Mourelatos *et al*., 1990 Gonatas *et al*., 1992
Cytosol	Ubiquitin positive aggregates	Kieran *et al*., 2007 Hetz *et al*., 2009
	EDEM upregulation (ERAD)	Hetz *et al*., 2009
Autophagy	LC3-II levels increase	Hetz *et al*., 2009
	ATG5-12 levels increase	Hetz *et al*., 2009
	BECLIN-1 upregulation	Hetz *et al*., 2009

F. ER abnormalities in fALS induced by SOD1 mutations

Approximately 20% of the typical fALS (adult-onset) cases are linked to more than 100 dominant mutations in the gene encoding for the antioxidant protein SOD1 (*Als1*), the best-characterized form of fALS (20, 248, 266). These mutations trigger misfolding and abnormal intracellular aggregation of SOD1, which is associated with neuronal dysfunction and

cytotoxicity. Mice and rats expressing human fALS-linked mutant SOD1 develop a motoneuron disease that is symptomatically and pathologically similar to human ALS, suffering paralysis, motoneuron degeneration, and age-dependent protein aggregation (20, 245, 248). Interestingly, mutant SOD1 variants with a higher propensity to aggregate are associated with a faster and more aggressive pathology in humans (41, 265, 343), in addition to animal (59, 132) and cellular (248) models of the disease. In contrast, other correlative studies have suggested that SOD1 aggregation may not be directly associated with neurotoxicity (see examples in 261, 301, 346, 347). A recent report on a *C. elegans* fALS model (85) also described that the toxicity of different mutant SOD1 variants may not be explained by their misfolding/aggregation, but rather by influence on genetic interactions related to genetic backgrounds (85). Increasing evidence suggests that soluble small oligomers may operate as the more neurotoxic species in fALS (141, 363), similar to findings described in Alzheimer's disease and other related pathologies (see reviews in 246, 290, 320). Intense research is needed to clarify the exact relationship between mutant SOD1 aggregation and its toxicity. However, the data discussed in this review suggest a more complex scenario where mutant SOD1 alters essential cellular processes related to protein folding, leading to a drastic alteration to general proteostasis. Consequently, unfolded proteins accumulate, triggering chronic stress and neuronal dysfunction.

Upregulation of UPR markers have been described in different mutant SOD1 transgenic mice, observing activation of the three major UPR signaling branches (Fig. 3 and Table 1) (2, 149, 228). An early report described the upregulation of ATF4 in addition to activation of ATF6, IRE1α, and *xbp1* mRNA splicing in spinal cord of symptomatic $SOD1^{G93A}$ transgenic mice (149), and these data have been confirmed by many groups (2, 131, 148, 217, 228, 284, 342, 351). A recent elegant study performed a systematic analysis of ER stress markers on two subgroups of motoneurons by laser microdissection from several mutant SOD1 transgenic mouse models: a group of neurons that die early during the course of the disease and a second group that is resistant to the disease. This study showed that only affected motoneurons of fALS mouse models were selectively prone to ER stress. Furthermore, a large set of ER stress markers were observed from birth forward in mutant SOD1 transgenic mice, with a clear activation a month before the earliest denervation detected during the asymptomatic phase of the disease (284). In this study, accumulation of ubiquitin-positive inclusions was observed in both vulnerable and resistant motoneurons, suggesting that protein misfolding occurs only in affected neurons presenting early chronic ER stress (284). Moreover, a proteomic analysis of spinal cord tissue from transgenic mice expressing human $SOD1^{G93A}$ revealed that the most induced proteins in symptomatic animals were the UPR target genes *erp57* and *pdi*, placing the UPR as a major signaling pathway activated in the disease (2). These data have been also recapitulated in cellular models of fALS where the overexpression of SOD1 mutants activate the UPR, leading to ER stress-mediated apoptosis (2, 121, 232, 319, 354).

Although wild-type SOD1 is a cytosolic protein synthesized by free ribosomes and lacks a classical organelle-targeting sequence, a subpopulation of both wild-type and mutant SOD1 are also observed at the ER and GA compartments (149). Biochemical and histological studies revealed that a fraction of insoluble-high molecular weight species of mutant SOD1 accumulates inside these two organelles *in vivo* as shown by subcellular fractionation and immunofluorescence analysis (2, 149, 332, 335). Cell-free translocation assays provided evidence indicating that monomeric SOD1 is a

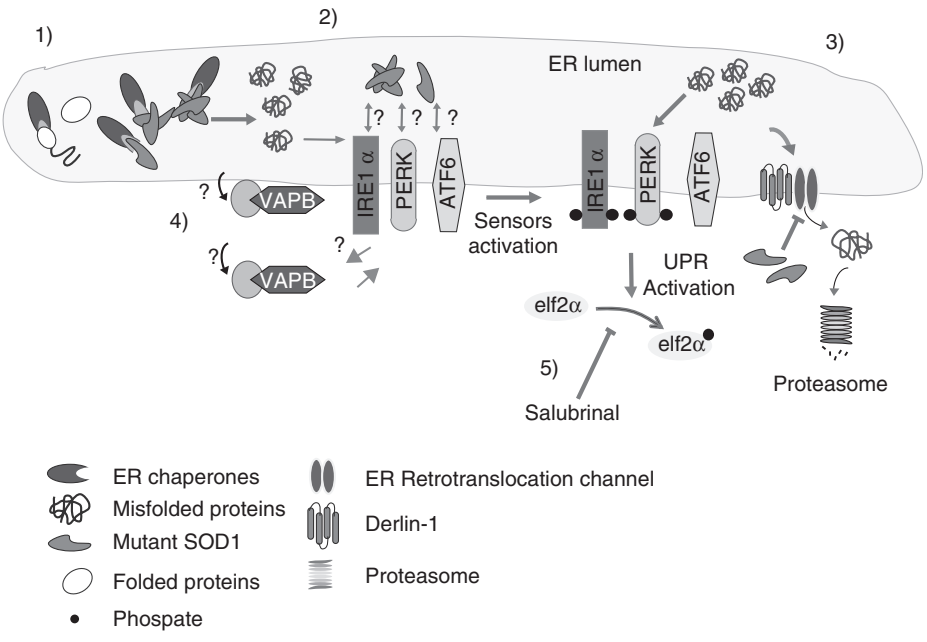

FIG. 3. ER stress and fALS. Various models to explain the occurrence of ER stress in fALS have been proposed. Under normal conditions, ER foldases and chaperones maintain the correct folding status at the ER. The presence of mutant SOD1 at the ER lumen may activate the UPR by different mechanisms: a) the recruitment or mislocation of chaperones and foldases through interactions with mutant SOD1 (i.e., PDI and BiP/GRP78) may impair protein folding of other ER proteins; b) The interaction between mutant SOD1 and DERLIN-1 could block ERAD activity, leading to accumulation of ERAD substrates at the ER lumen, inducing ER stress; c) VAPB, a protein that functions in vesicle transport between the ER and GA, modulates the UPR possibly through a direct interaction with signaling components; and d) Salubrinal, a small molecule that modulates the PERK pathway, gives protection against the disease by alleviating protein misfolding stress, suggesting that the attenuation of unfolded protein load may be beneficial in ALS.

molecular form that can translocate into luminal structures in the presence of ATP, suggesting an active redistribution process of the protein (332). It has been hypothesized that mutant SOD1 translocation from the cytosol into the ER occurs through the exposition of hydrophobic patches (332), but the mechanism explaining the presence of SOD1 at the ER lumen is not well understood.

Chemical cross-linking studies also revealed an age-dependent aggregation of mutant SOD1, but not of wild-type SOD1, prominently in ER-enriched fractions (332). Remarkably, a direct interaction between mutant SOD1 and PDI or with BiP/GRP78 was observed in microsomal fractions of spinal cord extracts (2, 149). Similarly, a co-localization between SOD1 aggregates and PDI has been observed through histological analysis. These observations correlate with the finding indicating that mutant SOD1 oligomers are possibly generated by abnormal intra- and intermolecular disulfide bonds through oxidation of two free cysteines on the SOD1 tertiary structure (see Fig. 6) (9, 36, 59, 75, 82, 141, 230). In favor of this idea, mutation in cysteines involved in inter disulfide bond formation abrogates mutant SOD1 aggregation and neurotoxicity (36, 230). In addition, *in vitro* studies demonstrated that inhibition of PDI activity with bacitracin enhances the accumulation of mutant SOD1 aggregates (2, 342), and ectopic expression of PDI decreases ER stress induction and toxicity induced by mutant SOD1 expression (342). Interestingly, a small molecule mimicking the protein disulfide isomerase active site protected against mutant SOD1 inclusion formation (342). Finally, it was recently shown that a member of the reticulon family of proteins, Reticulon-4A (NOGO-A), is a novel regulator of PDIs (359). Using genetic approaches, the authors demonstrated that expression of NOGO-A provides protection against fALS, correlating with a redistribution of PDI into a punta pattern (359). Deletion of a single copy of the NOGO-A gene (*rtn4*) accelerates disease onset and progression, while deletion of both copies further worsens disease. Previously, Dupuis and co-workers identified increased levels of NOGO-A at early asymptomatic stages in ALS transgenic mice in addition to both postmortem and biopsy samples from ALS patients (66). Overall, these data suggest a protective role of NOGO-A on disease progression, which may be associated with PDI function. However, most of the studies linking PDIs and other ER chaperones with fALS *in vivo* are correlative and the potential role of PDIs in the pathogenesis of disease remains to be established with direct studies using genetic or pharmacological manipulation.

A recent report evaluated the possible effects of decreasing ER stress levels in ALS using pharmacological approaches. Salubrinal, a small molecule described to modulate the PERK pathway (22), was shown to suppress aggregation of mutant SOD1 as well as SOD1 toxicity on a neuroblastoma cell line (232). Salubrinal selectively engages the translational control functions of the UPR by inducing eIF2α phosphorylation through inhibition of its phosphatase (22). Remarkably, treatment of mutant SOD1 transgenic mice with salubrinal leads to significant protection against disease progression, associated with an improvement of muscle strength and a significant increase in lifespan. The protective effects of salubrinal treatment correlated with attenuated levels of ER stress in vulnerable motoneurons and decreased glial activation (284). These results demonstrated for the first time a causal role of ER stress in ALS *in vivo*, suggesting a potential beneficial effect of targeting the PERK/eIF2α pathway to alleviate protein folding stress in ALS (284). Small compounds termed chemical chaperones have been shown to stabilize protein structure, attenuating ER stress levels in many different disease models (see examples in 145, 237, 262). The chemical chaperone 4-phenylbutyrate (4-PBA) decreases motoneuron apoptosis in mutant SOD1 transgenic mice, improving motor performance and lifespan. However, the effect of this drug on ER stress levels and its activity as a chemical chaperone in the ALS model employed was not tested in the study (232). Chemical chaperones such as tauroursodexycholic acid (TUDCA) and 4-PBA have been shown to decrease ER stress levels in models of diabetes (237), and brain ischemia (262), in addition to protect against neurodegeneration and protein aggregation in Huntington's disease mouse models (145). A chemical-chaperone approach to treat ALS remains to be systematically evaluated.

Our group recently investigated the contribution of ER stress to ALS using a genetic approach (121). We knocked down components of the three major UPR branches in a cellular model of fALS. Reduction in the expression levels of ATF4 and ATF6 increased the rate of mutant SOD1 aggregation, consistent with the common idea indicating that the UPR has a protective role in attenuate unfolded/misfolded protein accumulation (186). Reduced expression of IRE1α or XBP1 unexpectedly reduced the generation of mutant SOD1 aggregates and improved survival of cultured motoneurons. To test the role of XBP1 in fALS *in vivo*, we employed a conditional knockout mouse for xbp1 that we recently generated together with Laurie Glimcher's laboratory (117). XBP1 deficiency in the nervous system bypassed the embryonic lethality observed in the full knockout mice (259) without any spontaneous disease phenotype and did not affect the progression of a prion disease model (117). Using this mouse model, we generated mutant SOD1 mice with xbp1 deleted specifically in the nervous system (121). Similar to our cellular studies, these mice exhibited delayed ALS disease onset and increased lifespan in female mice, uncovering an unexpected beneficial effect of targeting the IRE1α branch of the UPR. Both cellular and *in vivo* approaches revealed an enhancement of mutant SOD1 clearance due to increased levels of autophagy in motoneurons, a cellular pathway involved in the degradation of mutant protein aggregates in many neurological diseases (121) (see below for detailed discussion).

G. Chronic ER stress and apoptosis in fALS

The predicted functional significance of ER stress in the disease process is complex due to two paradoxical interpretations. First, activation of the UPR could result in protective responses to increased protein folding and quality control mechanisms (the adaptive phase). On the other hand, ER stress may represent a deleterious signaling event during chronic stress due to an irreversible disturbance of ER homeostasis (pro-apoptotic phase). Many groups have described upregulation of ER stress related pro-apoptotic factors in the spinal cord of late and end stage disease SOD1 animals and sALS-derived human patient samples, suggesting the occurrence of chronic or irreversible ER stress (Tables 1 and 2). For example, the upregulation of the pro-apoptotic gene gadd153 (CHOP), has been described by different groups in

fALS mouse models (131, 341). Processing of pro-caspase-12 was described for the first time by Wootz and co-workers in the spinal cord of mutant SOD1 transgenic mice (352). Additionally, processing of pro-caspase-12 or pro-caspase-4 in rodents and humans, respectively, was correlated with motoneuron loss in ALS by others groups (2, 341, 351, 352). The contribution of caspase-12 to ALS has not been addressed yet. We have described the activation of caspase-12 in animal models of prion-related disorders, observing a close correlation with the extension of neuronal loss in different brain areas (118, 119). However, when we addressed the functional role of caspase-12 during the prion disease process using caspase-12 knockout mice, no alterations on disease progression or neuronal loss were observed (303). A similar study is needed in ALS mouse models.

Alternatively, ER stress-inducible BCL-2 pro-apoptotic genes, including *bim* and *puma*, are upregulated in symptomatic mutant SOD1 transgenic mice (120, 148). We have performed a systematic analysis to quantify the expression levels of the majority of BCL-2 family members in the spinal cord of symptomatic fALS mice and monitored the expression of the mRNA of the BH3-only proteins BIM, BMF, BAD, PUMA, NOXA, BID, and BIK, in addition to the pro-apoptotic BAX and BAX, and anti-apoptotic proteins (120). From the set of pro-apoptotic BCL-2-related genes analyzed, we observed a marked upregulation of *bim* mRNA, and a slight increase in the mRNA levels of *bid*, *puma*, and *noxa*. At the protein level, we confirmed the induction of BIM and PUMA in the spinal cord of two different mutant SOD1 transgenic mouse models at the symptomatic stage (120). No changes on BAX or BID protein expression were observed in the same experiments. To test the possible contribution of BIM to fALS pathogenesis, we crossed *bim* knockout mice with a mutant SOD1 transgenic model and demonstrated that the ablation of *bim* expression lead to a slight delay in disease onset and increased lifespan, likely due to a marked decrease of cellular apoptosis in the spinal cord ventral horn (120). The pro-apoptotic function of *bim* was also observed by knocking down its expression in cellular models of fALS using RNAi approaches and mutant SOD1 expression (120).

Another report confirmed the upregulation of BIM and PUMA expression in a mutant SOD1 mouse model (148). In this work, the specific contribution of *puma* was assessed to the disease process. Analysis of the lifespan of cross-bred mice demonstrated that deletion of *puma* in $SOD1^{G93A}$ mice did not significantly improve survival. However, further analysis showed a significant delay in disease progression in *puma* knockout mice (148). Disease progression was monitored using functional assessments of motor performance and body weight loss. At 90 days, sciatic motoneuron survival of *puma* deficient-$SOD1^{G93A}$ mice increased significantly compared with $SOD1^{G93A}$ littermates. Gliosis and microglial activation were also reduced in *puma* deficient mice (148). Taken together, activator BH3-only proteins BIM and PUMA are important mediators of neuronal apoptosis in fALS mouse models.

Early studies demonstrated that overexpression of BCL-2 through various strategies as well as deletion of *bax*, two components of the core apoptosis machinery, significantly affected disease onset in experimental fALS models (6, 93, 356, 357). Interestingly, Robert H. Brown's group described a physical interaction between mutant SOD1 and BCL-2 at the mitochondrial membrane, and it was proposed as an interesting mechanism to trigger apoptosis due to inactivation of the anti-apoptotic function of BCL-2 (242). The selective induction of the anti-apoptotic protein BCL2a1 was also reported in fALS motoneurons in mouse models with a high degree of specificity. However, its possible functional role on ALS has not been addressed directly (39, 249). Although significant beneficial effects are reported by targeting the BCL-2 family of proteins in fALS models, the contribution of the apoptosis process to the disease is in general minor, and only prolongs mouse survival for a few days. In addition, the role of apoptosis as a death mechanism in ALS is still debated (17, 148, 191, 228). Alteration of motoneuron function and nerve degeneration is proposed to be a more critical event leading to ALS-related pathology. Then, cell death may be a secondary event related to irreversible damage to motoneuron function.

H. Blocking the first exit: ERAD dysfunction in fALS

Activation of the UPR transcription factor XBP1s controls the upregulation of a subset of genes related to protein quality control, ER/GA biogenesis, and ERAD (Fig. 1) (264). One of the known XBP1s target genes is *edem1*, an essential component of ERAD. As mentioned, we have described that *xbp1* deficiency triggers autophagy-mediated degradation of mutant SOD1 (121). This phenotype was also associated with decreased ERAD activity when *xbp1* was knocked-down in a motoneuron cell line. Similarly, decreasing the expression of *edem1* lead to autophagy-mediated degradation of mutant SOD1 (121). We proposed that the accumulation of abnormally folded proteins at the ER due to mild ERAD impairment might operate as a compensatory signal to trigger autophagy and recover homeostasis (194). A similar model was proposed to explain the occurrence of ER stress in models of Huntington's disease (270). However, the connection provided between ERAD and the upregulation of autophagy activity by *xbp1* deficiency was correlative and different alternative models to explain the engagement of autophagy remain to be tested (see discussion of models in 194).

Of note, a novel pathogenic mechanism of mutant SOD1-induced ER stress was recently proposed. The cytosolic pool of mutant SOD1 was shown to inhibit ERAD activity related to decreased retro-translocation of ERAD substrates to the cytosol, correlating with the induction of ER stress (228). SOD1 mutants specifically interact with the cytoplasmic region of DERLIN-1, an essential component of the ERAD machinery (Fig. 3) (228). This interaction resulted in impaired ERAD activity, leading to ER stress possibly due to the accumulation of ERAD substrates at the ER lumen. The authors showed that knocking down *derlin-1* or *derlin-2* completely inhibited *xbp1* mRNA splicing and the activation of the IRE1α/JNK pathway in the context of ALS. Similarly, biochemical disruption of the SOD1/DERLIN-1 complex protected from mutant SOD1-mediated neurotoxicity (228), suggesting a critical role of this interaction in the occurrence of ER stress and motoneuron loss in fALS cellular models.

In the same study, ERAD impairment in fALS models was linked to the engagement of ER stress pro-apoptotic events such as ASK1/JNK activation (228). Remarkably, *ask1* deficient mice were markedly protected against SOD1 pathogenesis *in vivo* (228). The mean survival of mutant SOD1 mice

was increased by more than 4 weeks when *ask1* expression was ablated. However, disease onset was not significantly affected. The protective effects of *ask1* deficiency correlated with an enhancement of motoneuron survival in symptomatic mice. Similarly, the expression of the ERAD-related ubiquitin ligase C-terminus of HSP70 interacting protein (CHIP) attenuates the neurotoxicity of mutant SOD1 *in vitro*. These effects were due to SOD1 ubiquitination followed by proteasomal-mediated degradation (130, 229, 333). Mutant SOD1 interacts with HSP/HSC70 *in vivo* (333), and ubiquitinated protein inclusions in spinal cord motoneurons of $SOD1^{G93A}$ transgenic mice are CHIP-immunoreactive (333).

Although the study from Ichijo's group suggested a critical role of ERAD impairment on the induction of ER stress and motoneuron death in fALS (228), the potential contribution of the ER-located pool of mutant SOD1 to the occurrence of ER stress remains to be determined. Since aggregated mutant SOD1 physically interacts with essential ER luminal chaperones (i.e., PDI and BiP), this association may affect chaperone activity by an abnormal recruitment mechanism. Alternatively, a direct recognition of misfolded mutant SOD1 by UPR stress sensors at the ER lumen could also provide a possible explanation for the engagement of ER stress responses in fALS models. Finally, other mechanisms of ER stress engagement are open for investigation, including alterations in calcium homeostasis, lipid metabolism, or inhibition of vesicular trafficking.

I. ER stress in fALS induced by VAP mutations

Zatz and co-workers identified a new locus for ALS/motoneuron disease at 20q13.3 (*als8*) in a large Brazilian family (202), caused by a missense mutation in the *vesicle-associated membrane protein (VAMP)/synaptobrevin associated membrane protein B* gene ($VAPB^{P56S}$ protein) (227). *Vapb* encodes a ubiquitously expressed homodimer, which belongs to a family of intracellular vesicle-associated membrane-bound proteins that presumably regulate vesicle transport between the ER and GA (243, 298). VAPB function is closely related to the traffic of several lipid-binding proteins through the secretory pathway (138, 173). $VAPB^{P56S}$-associated pathology is characterized by tubular VAPB aggregates that are in close proximity to the ER (317). Expression of mutant VAPB or knocking down *vapb* in primary neurons causes distortion to the GA network and cell death (317). $VAPB^{P56S}$ expression leads to motoneuron degeneration possibly via a dominant-negative mechanism whereby mutant aggregates may trap wild-type VAPB, thus impairing its lipid-binding protein function (317). Moreover, the $VAPB^{P56S}$ mutation was shown to directly affect ATF6- and XBP1-dependent transcriptional responses (88). A specific domain of VAPA and VAPB was found to physically interact with ATF6 at the ER. Overexpression of VAPB attenuates the activity of ATF6/XBP-1-mediated transcription, an effect enhanced by the P56S mutation (88). These data indicate that VAPB proteins may directly interact with components of UPR signaling. Consistent with these observations, expression of $VAPB^{P56S}$ increases the vulnerability of NSC34 motoneuron cells to ER stress-induced death (312). A reduction in the expression of VAPA and VAPB has been described in tissue from some sALS cases, in addition to mutant SOD1 transgenic mice (317), suggesting a wider role for VAPB in sporadic and familial forms of ALS. More research is needed to better address the pathogenic mechanism of $VAPB^{P56S}$.

J. TDP-43, FUS/TLS, and abnormal mRNA metabolism in ALS

Accumulating evidence indicates that alterations in proteins related to mRNA metabolism is a salient feature of ALS pathogenesis (46, 309), a phenomena also linked to protein misfolding. For example, abnormal subcellular distribution and cytoplasmic aggregation of TDP-43 is widely reported in sALS and fALS cases, in addition to frontotemporal lobar degeneration (223, 345). TDP-43 regulates different processes related to gene expression, including transcription, splicing, and mRNA stability through a RNA and DNA binding activities (Fig. 4) (46, 87, 309). A recent proteomic analysis of the TDP-43 interactome revealed associations with proteins related to RNA metabolism (78), and disease-related TDP-43 mutants were not affected in their interaction profile. Remarkably, TDP-43 interacts with components of *stress granules*, in addition to be itself recruited to these subcelular structures (78). Similarly, oxidative stress induces TDP-43 redistribution to *stress granules* (33), however the biological relevance of these observations is not clear yet. TDP-43 was found in ubiquitinated protein inclusions, and it was shown to be hyperphosphorylated and ubiquitinated. Most ALS-linked mutations in TDP-43 are mapped to the C-terminal glycine-rich region, which is involved in protein–protein interactions between TDP-43 and other ribonuclear proteins (162). Of note, C-terminal fragments of TDP-43 are specifically accumulated in ALS and frontotemporal lobar degeneration-derived tissue, and these fragments have a high tendency to aggregate into intracellular inclusions (223).

Mice expressing a disease-linked mutant form of human TDP-43 develop a progressive and fatal neurodegenerative disease reminiscent of ALS, showing motoneuron loss, motor impairment, muscular atrophy, and axonal degeneration (345). Remarkably, this mouse model present pathologic ubiquitinated protein aggregates that accumulate only in specific neuronal populations in the frontal cortex and the spinal cord. These results suggest that expression of TDP-43 mutants alter general proteostasis, resulting in accumulation of misfolded proteins (345). However, the role of ER stress in the pathogenesis of TDP-43 has not been addressed. A zebrafish model to study TDP-43 function was also recently generated (136). Expression of disease-related human TDP-43 as well as the wild-type protein, albeit to a lesser extent, caused a motor phenotype in zebrafish embryos consisting of shorter motoneuron axons, premature and excessive branching, as well as movement deficits (136). Remarkably, knockdown of zebrafisfh TDP-43 with morpholinos lead to a similar phenotype, which was rescued by the ectopic expression of wild-type human TDP-43 and not with the ALS-linked mutants (136). This data suggested that both a toxic gain of function due to protein aggregation/mislocation together with a loss of the normal biological function upon mutations contribute to disease pathogenesis.

TDP-43 shuttles between nucleus and cytoplasm in a transcription-dependent manner (4). The C-terminal region of TDP-43 is essential for its cellular localization since its deletion results in the formation of large nuclear and cytoplasmic aggregates (4). A recent report suggested that

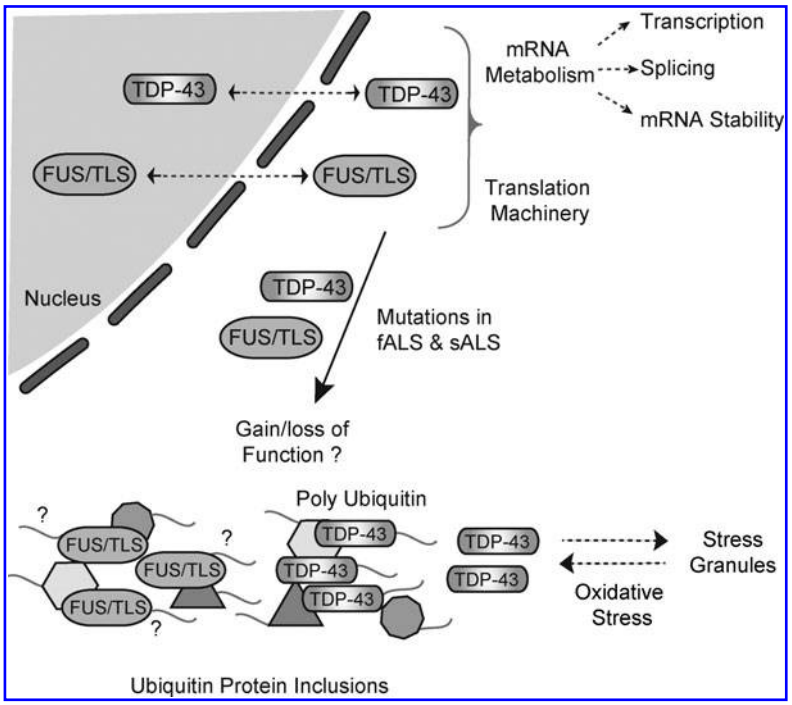

FIG. 4. TDP-43 and FUS/TLS in ALS. Some familial and sporadic forms of ALS are linked to mutations of TDP-34 and/or FUS/TLS. Both proteins have been implicated in several steps of related to the regulation of gene expression, including transcription, RNA splicing, RNA transport, and translation. TDP-43 and FUS/TLS normally shuttle between the nucleus and the cytoplasm. Mutant forms of these proteins accumulate in the cytosol and have been found in ubiquitin-positive protein inclusions. TDP43 itself has been shown to be poly-ubiquitinated. Additionally, under oxidative stress conditions, TDP-43 can be found in stress granules. It is not clear if TDP-34 and FUS/TLS mutations lead to ALS due to a loss of their biological function or the acquisition of neurotoxic activities upon mutations.

the specific toxicity induced by mutant TDP-43 expression was actually associated with increased cytoplasmic mislocalization of TDP-43 (12). Inclusion bodies were not necessary related to neurotoxicity, but the amount of cytoplasmic TDP-43 was a strong and independent predictor of neuronal death (12).

Another ALS-linked gene is also related to mRNA metabolism. Recent studies identified 13 mutations in the *fused in sarcoma/translated in liposarcoma* (*Fus/Tls*) gene on chromosome 16 that were specific for fALS (160, 336). FUS/TLS is involved in the regulation of transcription, RNA splicing and transport, and it has functional homology to TDP-43. Expression of ALS-linked mutant FUS/TLS leads to accumulation of cytoplasmic protein aggregates and defective RNA metabolism (160, 336) (Fig. 4). Mutations in *fus/tls* were recently observed in some sALS cases, suggesting a broad role of FUS/TLS in ALS pathogenesis (13, 35, 314). Although a connection has been provided between alteration in mRNA metabolism and protein misfolding in ALS, it remains to be determined if ER stress and oxidative damage are also relevant factors in TDP-43- and FUS/TLS-mediated ALS.

In addition to *Tdp-43* and *Fus/tls*, other reports have associated ALS-liked genes to aberrant RNA metabolism including *Sod1*, *Angiogenin* and *Senataxin* (see review in 309). We envision an emerging model where an interconnection between altered mRNA metabolism and abnormal *proteostasis* emerges as a key player in ALS pathogenesis.

K. Oxidative protein damage in ALS

Mitochondrial dysfunction had been extensively studied as a central contributor to ALS pathogenesis, possibly due to the generation of oxidative stress (see specialized reviews in 8, 67, 113, 114). Since this article focuses in secretory pathway stress and protein misfolding, we will summarize some relevant aspects about oxidative stress in ALS that are related to alterations of proteostasis in the disease. Transgenic mouse and cell culture models of ALS based on the expression of mutant SOD1 recapitulate the oxidative damage to protein, lipid, and DNA observed in human patients (100). Several studies have reported increased oxidative stress in sALS postmortem tissue and elevated protein carbonyl levels in the spinal cord and motor cortex (see examples in 73, 74, 294). Additionally, increased 3-nitrotyrosine levels, a marker for peroxynitrite-mediated damage, was found in spinal cord motoneurons of both SOD1-mediated fALS and sALS cases (21). Therapies with antioxidants such as vitamin E, selegiline, N-acetylcisteine, and coenzyme Q10 have shown potential beneficial effects in ALS animal models. However, as other human clinical ALS trials, these treatments showed disappointing results in terms of improving ALS symptoms and delaying disease onset (see 11).

As mentioned before, many studies have shown that ALS-linked SOD1 mutants do not induce pathology through changes of its enzymatic antioxidant activity because some mutants are enzymatically inactive, others are hyperactive, and other mutants have similar activity to the wild-type protein (47, 243, 248, 298). However, most SOD1 mutants induce the pathology when they are expressed in transgenic mice, associated with abnormal SOD1 aggregation and motoneuron dysfunction (34, 47, 59). There are several mitochondrial-specific antioxidant mechanisms, including SOD1, SOD2 (manganese SOD), glutathione peroxidases, and peroxiredoxin 3, which are usually able to attenuate an abnormal rate of reactive oxygen species (ROS) production (302). Although SOD1 is generally found in the cytosol, it is also present within the mitochondrial intermembrane space (123, 180, 234, 311), associated with increased oxidative damage and decreased mitochondrial respiratory activity (75, 192).

ROS can be originated as a consequence of aerobic metabolism and, mostly due to leakage of electrons from the

mitochondrial respiratory chain, result in incomplete reduction of molecular oxygen during oxidative phosphorylation. This event generates the superoxide radical anion (O_2^-) and hydrogen peroxide (H_2O_2). A portion of cellular ROS is produced by cellular oxidative enzymes in the cytoplasm and the ER (Fig. 5). Oxidative stress can enhance various pathogenic events including an increase in intracellular Ca^{2+} levels related to glutamate excitotoxicity (75, 192). Increases in cytosolic calcium levels are buffered by mitochondria, augmenting ROS production, opening of the permeability transition pore, an event recently linked to ALS *in vivo* (Fig. 5) (142, 146, 190, 191). Alterations to mitochondrial morphology have been observed in motoneurons from ALS patients and animal models (157, 191, 283, 296, 349), in addition to decreased electron transport chain activity, altered mitochondrial membrane potential (29, 134, 192, 199, 313), disrupted calcium homeostasis (29, 313), increased mitochondrial DNA damage (191), and reduction in general mitochondrial antioxidant defense mechanisms (81, 350).

Notably, a gene expression profile study carried on motoneurons expressing mutant SOD1 reported the downregulation of genes involved in antioxidant responses, including the transcription factor *nrf2* (nuclear factor (erythroid-derived 2)-like 2), several members of the glutathione S-transferase family, and two peroxiredoxins (154). Moreover, reduced Nrf2 mRNA and protein levels have been reported in spinal cord neurons from ALS patients (282). Nrf2 is known to interact with promoter sequences containing the antioxidant-response element (ARE) sequence, increasing the expression of proteins involved in antioxidant defense systems (225). It is predicted that downregulation of Nrf2 expression in ALS may reduce the ability of cells to remove ROS, resulting in a gradual increase in oxidative stress over time.

The relationships between oxidative stress and ER stress in sALS was first suggested by a correlative study describing the co-existence of oxidative damage to ER proteins and chaperones with the occurrence of ER stress in the spinal cord of postmortem samples from sALS patients (129). These events were also correlated to increased accumulation of abnormal ubiquitin-positive protein inclusions (129). A recent study demonstrated the occurrence of early mRNA oxidation in spinal cord from ALS patients, in addition to pre-symptomatic mutant SOD1 transgenic mice (46). A group of mRNA was identified to be more susceptible to oxidation through a gene expression profile analysis of spinal cord tissue of pre-symptomatic $SOD1^{G93A}$ transgenic mice. These mRNAs encode proteins involved in essential cellular process related to protein biosynthesis (ribosome protein S6, eukaryote translation initiation factor 5), protein folding (heat shock protein 70KD protein 5, heat shock protein 90KDa alpha, class A member 1), ubiquitin-proteasome system (26S subunit ATPase2, proteasome subunit beta type 4, ubiquitin-conjugating enzyme E2D), and lysosomal function (lysosomal membrane glycoprotein 1) (46), suggesting that oxidative stress may lead to broad alterations to proteostasis (293). Of note, known fALS-related genes such as *als1/sod1, dctn1, and vapb* were also identified in the study (46).

Recent evidence in Parkinson's disease and Alzheimer's disease suggest that oxidation of ER chaperones is an important event in the pathology, triggering chaperone inactivation, and thus protein misfolding and ER stress (52, 55, 328). Nitrosylation of the cysteines present at the active site of PDI was shown to be a critical pathological event (52, 55). A recent

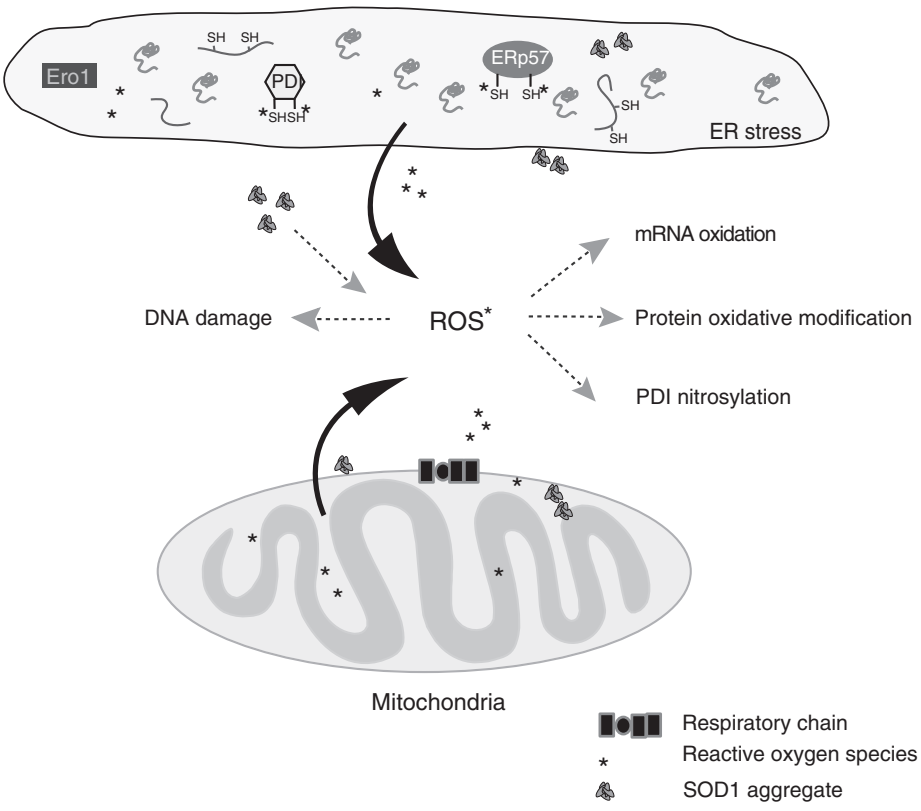

FIG. 5. Oxidative stress in ALS. In addition to the mitochondria, the ER is emerging as an important source of ROS under ER stress conditions. When the ER function is perturbed, an unbalance between the protein disulfide isomerases (i.e, ERp57 and PDI) and Ero1 redox functions generates excessive ROS as a result of disulfide bond formation. The generation of ROS has a broad impact on proteostasis initiated by modifications of key biological molecules including mRNA oxidation, protein modification, and chaperone inactivation, among other effects.

report from Julie Atkin's group described for the first time the occurrence of PDI nitrosylation in sALS-derived tissue (342). Increased levels of S-nitrosylated forms of PDI were observed in mutant SOD1 transgenic mice and human sALS spinal cord tissue, providing for the first time a direct link between general oxidative stress and ER stress/protein misfolding in the pathogenesis of ALS (Fig. 6) (342).

The ER can also operate as a source of oxidative stress, which is highly enhanced during sustained ER stress (see review in 276). When disulfide bonds are formed in proteins by PDIs, electrons are passed from thiol groups on the protein substrates to Ero1 and finally to molecular oxygen, resulting in the generation of ROS (200) (Fig. 6) (reviewed in 324). Any unstable disulfide bonds are reduced by glutathione in a reaction catalyzed by Ero1 that depletes cellular reservoirs of reduced glutathione and increases the number of thiol groups available for disulfide bond formation, consequently increasing ROS (44). Activation of the UPR increases Ero1 expression, which may reflect an imbalanced redox status at the ER (Fig. 6) (109). The actual contribution of the ER to the generation of ROS in ALS remains to be determined. In summary, increasing evidence indicates a relationship between oxidative stress and ER stress in the pathogenesis of ALS, possibly contributing to protein misfolding.

III. Second Stop: Golgi Apparatus

A. Golgi apparatus: Main functions

The Golgi apparatus (GA) is composed of a series of flattened parallel, interconnected cisternae organized around the microtubule-organizing center in the perinuclear region. The GA is a highly dynamic structure and plays a key role in the transport, processing, and targeting of proteins to their final destination, including the plasma membrane, ER, and lysosomes (70). In neurons, the GA is involved in the axoplasmic flow of numerous endogenous proteins and of exogenous macromolecules transported by orthograde, retrograde, and transynaptic routes (102, 260). Therefore, GA abnormalities may have detrimental consequences on axon and presynaptic terminal function (79).

Alterations in GA function or inhibition of ER-GA trafficking trigger ER stress (184). GA fragmentation and dispersal precede neuronal cell death induced by excitotoxins, oxidative/nitrosative insults, ER stress among other cell death stimuli (219). In addition, recent reports suggest the existence of specific cell death pathways initiated by irreversible GA damage, which may be downstream of ER stress in neurons (219). It has been also reported that during apoptotic cell death, Golgi stacks disperse and disassemble into tubulovesicular clusters (184). Interestingly, GA fragmentation during apoptosis is proposed to be independent to alterations in the cytoskeleton, suggesting an intrinsic GA-regulated process (211). Of note, the pro-apoptotic protease caspase-2 is localized in the GA and the nucleus. *Caspase-2* -deficient mouse fibroblasts are resistant to apoptosis induced by drugs that activate secretory pathway stress (188). Caspase cleavage of different GA proteins have been implicated in apoptosis (51, 165, 166, 182, 185, 188), suggesting an important role of GA-resident proteins in the initiation and fine regulation of stress responses and cell death signaling, in addition to the execution of organelle disassembly during apoptosis.

B. GA fragmentation in sALS

Fragmentation of the GA has been extensively reported in anterior horn motoneurons of spinal cord tissue derived from sALS patients and other neurological disorders (see examples

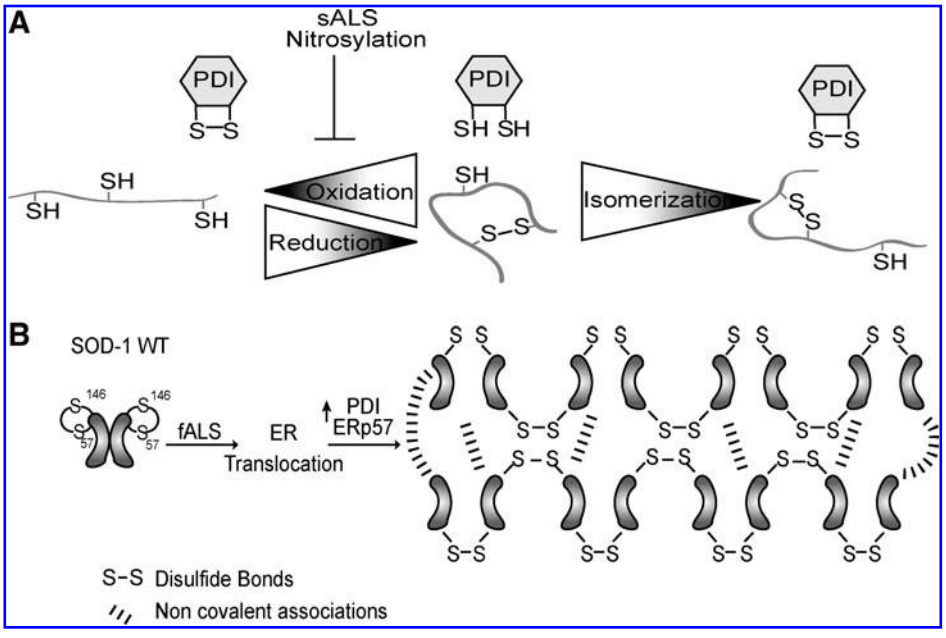

FIG. 6. PDI function and mutant SOD1 aggregation. (**A**) Protein disulfide isomerase (PDI) catalyzes the formation and breakage of disulfide bonds between cysteine residues during proteins folding at the ER. Generation of excessive reactive oxygen or nitrogen species results in protein misfolding due to PDI inactivation by S-nitrosylation of the active site of the enzyme. (**B**) fALS linked to mutations of SOD1 is characterized by the accumulation of stable SOD1 aggregates in motoneurons. Abnormal disulfide bonds formed between cysteines at positions 6 and 111 of different SOD1 monomers have been proposed to mediate the generation of oligomeric polymers at the ER, leading to the generation of large mutant SOD1 aggregates. Protein disulfide isomerases such as ERp57 and PDI may be involved in decreasing this pathological process.

in 80, 91, 209, 305). Namely, GA fragmentation refers to a loss of the normal network-like configuration, which is replaced by disconnected vesicular pattern (Tables 1 and 2) (40,90). In an early study, Gonatas and co-workers performed histopathological analysis of tissue from a small group of sALS cases (91). This study indicated that approximately 30% of motoneurons in five sALS cases showed a fragmented GA, whereas only ~ 1% of motoneurons from seven control with neurologic or systemic disease showed a similar change. Analysis of the distribution of cytoskeleton proteins did not depict any differences in the staining of neurons with fragmented or normal GA (91), suggesting that the morphological alteration of the organelle are not secondary to a gross lesion of the cytoskeleton. Similarly, other studies have shown that neurons with abnormal TDP-43 immunoreactivity are associated with dysfunction on GA morphology in sALS (79).

Interestingly, intrathecal injections of CSF from sALS patients into rodents using pup-mediated delivery caused marked fragmentation of the GA in spinal cord motoneurons (253). This phenotype was widely spread through the spinal cord, suggesting a putative transmissible pathological element present in CSF from these sALS patients in addition to an intrinsic sensitivity of motoneurons in accumulating GA-related defects (253). Whether GA network impairment in sALS is an indicator of pathogenesis or is representative of a disease consequence remains to be determined.

C. GA alterations in fALS-induced by SOD1 mutations

Similar to sALS, fragmentation of the GA is a consistent neuropathological feature observed in motoneurons from pre-symptomatic mutant SOD1 transgenic mice (Figs. 7 and 8) (210). In addition, GA pathology is enhanced with disease progression (306, 307). Interestingly, this event correlates with an impairment of secretory activity as measured by monitoring the transit of CD4 to the cell surface, a glycoprotein processed through the GA (308).

Wild-type SOD1 is secreted to the extracellular space, but the efficiency of secretion is decreased for most ALS-linked SOD1 mutants (326). ATP-dependent secretion of wild-type SOD1 has been described using a variety of cell types other than motoneurons, including human hepatocytes, fibroblasts, neuroblastomas, thymic-derived cell lines (31, 205, 206), and mouse astrocytes (161). The presence of extracellular endogenous wild-type and mutant SOD1 was observed in CSF of transgenic fALS rats (214). Turner and co-workers suggested that a diminished secretion of mutant SOD1 was associated with intracellular toxic protein inclusions and GA fragmentation in a motoneuron cell line (326). SOD1 secretion was sensitive to brefeldin A treatment, suggesting a classical route of secretion. Interestingly, although secretion was observed in neuronal and non-neuronal cell lines, only neuronal cells were shown to be sensitive to mutant SOD1 toxicity (326). Enforced secretion through addition of an extracellular targeting sequence to mutant SOD1 attenuated cytoplasmic aggregates and toxicity in transfected motoneuron cell lines (326), suggesting that intracellular accumulation of mutant SOD1 has deleterious effects. The same study demonstrated that chronic intraspinal infusion of wild-type SOD1 significantly prolonged survival of transgenic rats expressing mutant SOD1-^{G93A} for approximately 25 days without affecting disease onset, measured by no effect on the decline of motor performance (326). This protective effect was reproduced by antioxidant therapy in transgenic fALS mice, similar to the results observed by the injection of SOD1-catalase, suggesting that mutations in SOD1 may decrease any neuroprotective activity of extracellular wild-type SOD1 (135).

Extracellular accumulation of mutant SOD1 has also been suggested as a potential pathogenic effect in fALS (Fig. 7) (335), where secretion of mutant SOD1 results in nonautonomous effects related to neuroinflammation and glial activation (335). Through a yeast two-hybrid screening of a cDNA library from total spinal cord of pre-symptomatic mutant SOD1^{G93A} mice, Urushitani and co-workers identified mutant SOD1 interacting partners. This study revealed a specific association between mutant SOD1 and chromogranins, an interaction not observed with wild-type SOD1. Chromogranins are soluble proteins transported through the trans-Golgi network (TGN), and are part of large dense-core vesicles (LDCV) related to regulated exocytosis in neurons and endocrine cells (316, 335). In fact, it was shown that the secretion of mutant SOD1 is partially regulated by chromogranin expression and occurs as a protein complex with SOD1 from motoneurons, astrocytes, and interneurons. Through cell culture experiments, it was demonstrated that secretion of mutant SOD1 triggers microglial activation. These events contributed to microgliosis and finally enhanced neuronal cell death, possibly due to inflammatory and oxidative stress components (335). These observations emphasize the relevance of the motoneuron milieu in ALS and support the non-cell-autonomous theory of the disease (32, 247, 292, 335). A partial co-localization of mutant SOD1 and chromogranins was observed in fALS mouse models by immunofluorescence and electron microscopy studies. This data was also confirmed through subcellular fractionation and purification of GA- and microsome-enriched fractions followed by co-immunoprecipitation experiments (335).

Due to the occurrence of SOD1 secretion *in vivo*, the possible therapeutic benefits of immunization protocols on fALS were tested, aiming to reduce the burden of extracellular SOD1 mutants in nervous tissue (331). The authors employed recombinant mutant SOD1 to immunize mutant SOD1^{G37R} transgenic mice, and observed protective effects associated with a delay of disease onset and an extension of lifespan for more than four weeks (331). Evidence for a clearance of SOD1^{G37R} species in the spinal cord of vaccinated mice was also provided. In addition, the authors showed that intraventricular infusion of purified anti-human SOD1 antibody had beneficial effects on SOD1^{G93A} transgenic mice (331). These results provided the first evidence for a possible role of extracellular mutant SOD1 in fALS pathogenesis.

D. A nonclassical secretory pathway for mutant SOD1

One report indicated that wild-type and mutant SOD1 is also secreted via exosomes. Exosomes are small lipid membrane microvesicles formed by fusion of multivesicular endosomes (MVEs) with the plasma membrane, followed by the release of their cargo into the extracellular environment (89). Initially, exosomes were thought to operate as a cellular mechanism for releasing unnecessary proteins, but recent studies have revealed further functions of exosomes in cell-to-cell communication. Proteins associated with

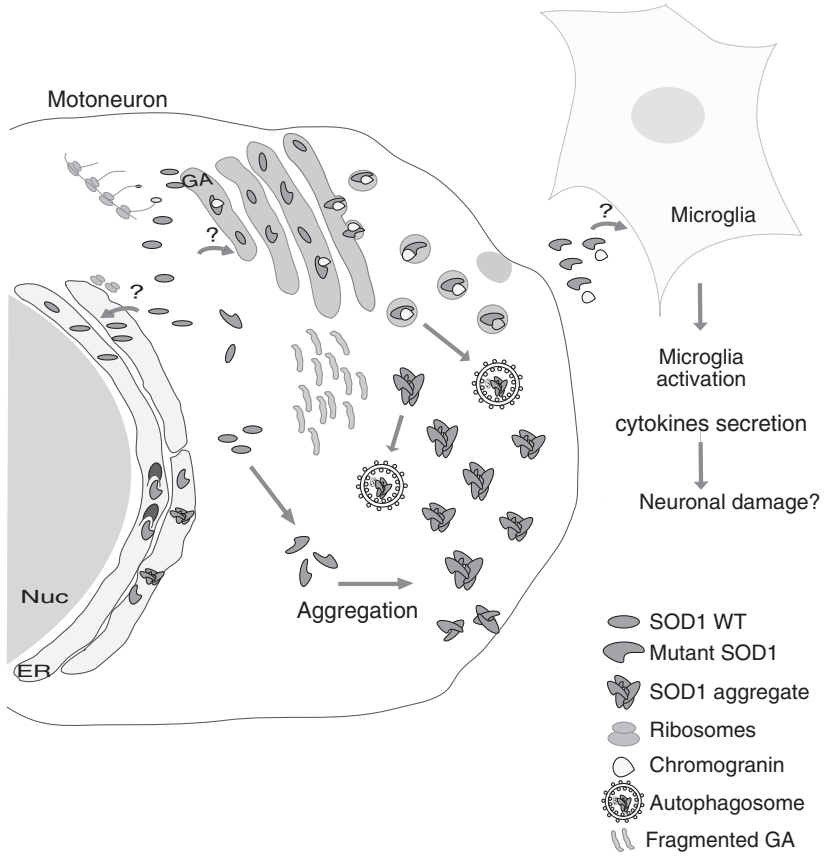

FIG. 7. Mutant SOD1 in the secretory pathway. Mutant SOD1 forms abnormal protein aggregates that are found in the cytosol, ER and GA. GA fragmentation has been observed in fALS and sALS motoneurons. In addition to wild-type SOD1, fALS-linked mutants are secreted to the extracellular space. This secretory route involves the translocation of SOD1 monomers to the ER by an unknown ATP-dependent mechanism, and then follows a classical secretory route. Mutant SOD1 physically interacts with chromogranins, an essential step for their secretion. Extracellular mutant SOD1 may have deleterious effects by inducing a drastic glial reaction, leading to the generation of neurotoxic species. In contrast, secretion of wild-type SOD1 may have protective effects due to its antioxidant activity. During its transit through the secretory pathway, vesicles containing mutant SOD1 may be targeted to lysosomal/autophagy mediated degradation.

neurodegenerative diseases, such as the prion protein and amyloid precursor protein (APP) can be selectively incorporated into MVE and subsequently released into the extracellular space within exosomes (338). Remarkably, the presence of exosome-containing proteins in body fluids has been proposed as an interesting candidate strategy for diagnosis of diseases such as brain cancer (338, 348). This nonclassical secretion pathway provides a potential explanation for extracellular prion spread and amyloid deposition, which may help understand the non-cell-autonomous aspects of ALS pathology and other neurodegenerative diseases (338).

E. GA disturbance and the Wobbler mouse

The first animal model of motoneuron degeneration was the *Wobbler* mouse, which was related to the occurrence of abnormalities in RNA metabolism (23). Motoneuron survival is severely compromised in the *Wobbler* mouse. However, the mutation responsible for the ALS-like phenotype in this mouse was later described as a missense mutation in *vacuole protein sorting 54* gene ($vps54^{L967Q}$) (286). VPS54 is a component of the GA-associated retrograde protein (GARP) complex of vesicle sorting of proteins (198), suggesting that VPS54 has an essential role in trafficking (286). Interestingly, the presence of intracellular ubiquitin inclusions and abnormal distribution of TDP-43 protein was revealed in the cytoplasm of neurons from *Wobbler* mouse. However, efforts to find mutations in the gene corresponding to chromosome 2p14-15 (related to *vps54*) after DNA sequencing of 96 individuals with sporadic ALS, 96 individuals with familial ALS, and 96 controls subjects lead to no conclusive results to consider *vps54* as an ALS–linked gene (198). Analysis of a larger number of patients is need to further address the role of VPS54 in ALS.

F. GA alterations in fALS induced by dynactin mutations

The dynactin complex has relevant functions in axonal retrograde transport. Several studies have described the occurrence of microtubule-based axonal transport abnormalities in mutant SOD1 transgenic mice (Fig. 8) (164, 176). Teulin and co-workers determined the consequences of targeting the dynein/dynactin complex in motoneurons in the context of ALS by generating transgenic mice with neuronal-specific expression of the N-terminus of bicaudal D2 (BICD2-N). BICD2-N is a motor-adaptor protein that is involved in dynein-mediated transport of cargoes (318). The fragment expressed of BICD2-N strongly binds and inhibits the dynein/dynactin complex (127). Interestingly, the expression of BICD2-N in motoneurons caused GA fragmentation and axonal neurofilament swellings (318). Unexpectedly, BICD2-N expression increased the lifespan of a fALS mouse model, suggesting that an unknown event related to dynein/dynactin-dependent transport may be relevant to ALS pathogenesis (147, 364).

IV. Third Stop: Endocytosis and Vesicle Transport

A. Endocytosis and multivesicular endosomes

Many cell-surface proteins, including receptors and bound ligands, are internalized via clathrin-coated pits and

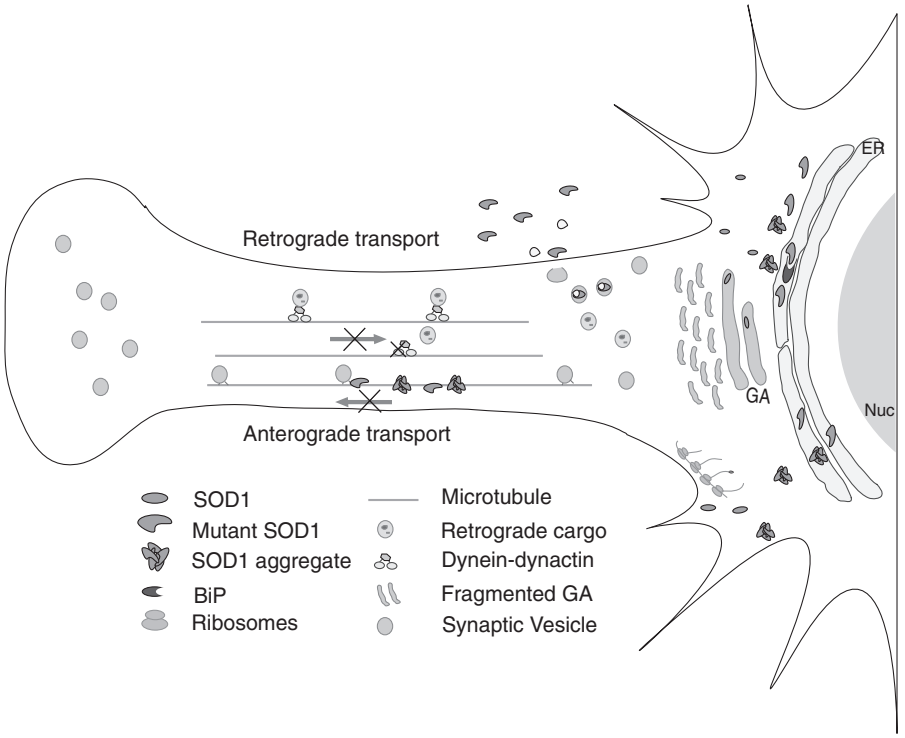

FIG. 8. Abnormal axonal transport in ALS. The dynein-dynactin complex constitutes essential motor proteins for retrograde fast axonal transport. Vesicles are loaded onto transport motors both in the cell body and the distal nerve terminal. Mutations in dynactin in humans and dynein in mice provoke motoneuron degeneration and motor impairment. Perturbation of neurofilaments through mutations and alterations in phosphorylation and the physical structure of the axon could also adversely affect axonal transport. In transgenic mouse models, mutant SOD1 impairs anterograde axonal transport.

successively transferred to early and late endosomes before delivery to lysosomes for degradation. During this process, proteins are also ubiquitinated as a signal for sorting into the luminal vesicles of MVEs, often referred to as 'multivesicular bodies' (MVBs) (96, 143). The formation of MVEs occurs by the inward vesiculation of endosomal membranes, a process driven by the endosomal sorting complexes required for transport (ESCRT)-0, I, II, and III. ESCRT-0, -I, and -II contain ubiquitin-binding subunits and mediate the sorting of the ubiquitylated cargo. Another component, ESCRT-III, is the principal machinery for the formation of intralumenal vesicles in MVEs (Fig. 9) (96, 143, 271). As a degradative sorting compartment, the MVEs are structural and functionally related with lysosome and autophagy-related vesicles (see below for detailed description of autophagy) (271). Deletion of different ESCRT subunits results in abnormal accumulation of autophagosomes and sometimes accumulation of cytoplasmic protein aggregates containing ubiquitinated proteins, suggesting a defect in autophagy-dependent degradative processes (76, 168). In the next section, we describe evidence linking the alterations to vesicular transport with three different genes associated with ALS.

B. Multivesicular endosomes in fALS

The most direct evidence involving the participation of MVEs in ALS comes from the identification of autosomal dominant mutations in the *Chmp2b* gene in a subset of fALS patients (Fig. 9) (239). *Chmp2b* gene codifies for the charged multivesicular body protein 2 B (CHMP2B) that is part of ESCRT III complex. Further studies demonstrated that the overexpression of mutant *Chmp2b* causes dominant-negative phenotypes associated with the accumulation of autophagosomes and intracellular protein aggregates, followed by neuronal death (76, 168, 272). Given the known role of basal autophagy in the homeostasis of the nervous system and the clearance of toxic misfolded proteins (104, 156), ESCRT dysfunction may be a relevant factor contributing to ALS and the alterations of proteostasis observed in motoneurons.

C. Vesicle transport and fALS: Alsin

Mutations in *als2,* the gene encoding ALSIN, have been linked to the onset of autosomal recessive motoneuron diseases, including juvenile-onset ALS, hereditary spastic paraplegia, and primary lateral sclerosis (99, 358). The primary structure of alsin is very complex, with many structural domains. Alsin is predicted to have three putative guanine regulators of chromosome condensation 1 (RCC1)-like domains (termed RLD); a diffuse B cell lymphoma (Dbl) homology/pleckstrin homology (DH/PH) domain; and a vacuole protein sorting 9 (VPS9) domain (45, 99). Alsin expression in neurons is observed in small punctuate pattern in dendrites, axons, cell body, membrane ruffles, and growth cones (322, 325). In addition, its expression has been reported to affect cytoskeleton dynamics through interactions with Rho subfamily members (325). Rho subfamily members such as Rac GTPases, Rho, and Cdc42, have been shown to regulate actin dynamics and neuritic outgrowth (61). Binding assays revealed a specific interaction between Rac1 and alsin through the DH/PH domains (322). It has also been shown that the VPS9 domain of alsin acts as an activator of GEF for the small GTPase Rab5, regulating endosome vesicle sorting (324). Together, these data suggest a role for alsin in axonal guidance in addition to the regulation of cytoskeleton and vesicle dynamics. Recently, alsin was demonstrated to exert a neuroprotective function via its Rho-GEF domain, inhibiting the neurotoxicity of mutant SOD1 *in vitro* (140). Although mutations in *als2* are associated with motor dysfunction in humans, mice deficient in *als2* have largely failed to recapitulate symptoms of motoneuron disease (26, 60, 98, 355). *als2* knockout mice appear to be normal, with a lifespan similar to wild-type

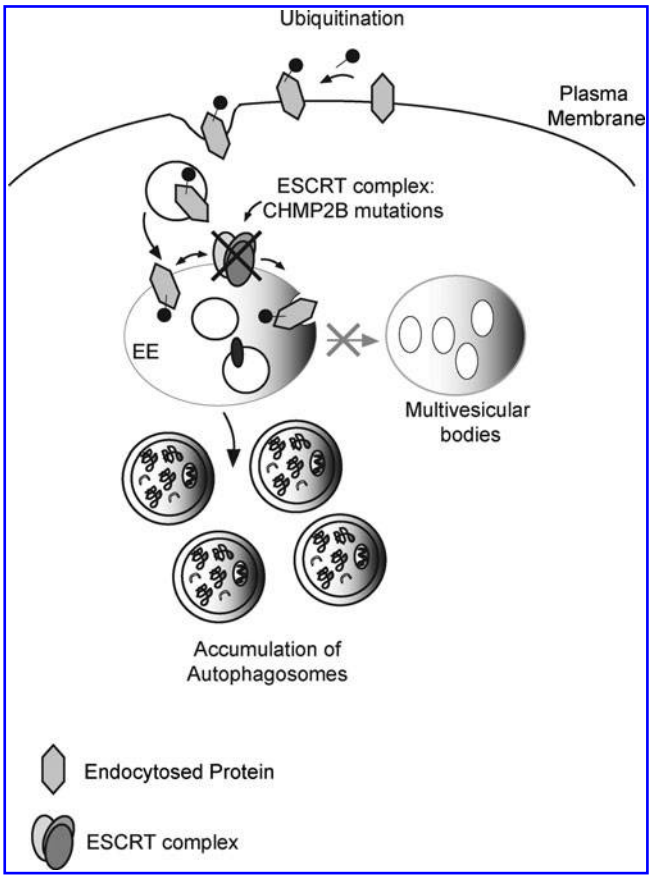

FIG. 9. An ALS mutation involved in multivesicular endosomes. Endocytosed proteins can undergo lysosomal-mediated degradation. This process requires sorting from early endosomes (EE) to mutivesicular endosomes by the endosomal sorting complex required for transport (ESCRT). Mutations in *chmp2b*, a component of the ESCRT complex, have been described in some ALS cases. Mutation in *chmp2b* induces an accumulation of autophagosomes and accumulation of protein aggregates, suggesting an alteration in vesicular trafficking and protein degradation mechanisms.

littermates (60). However, Guy Rouleau's group described two new animal models to address ALS2 function (95). Mice lacking exon 2 and part of exon 3 of *als2* developed mild signs of neurodegeneration compatible with the occurrence of axonal transport deficiency. Similarly, knockdown of *als2 in* zebrafish results in severe developmental abnormalities, swimming deficits, and motoneuron perturbation (95). The role of *als2* in ALS needs further investigation to solve these differences between animal models.

D. Vesicle transport and fALS: Dynactin

Retrograde axonal transport of cargoes such as vesicles, endosomes, signaling complexes, degradation products, and organelles all involve interaction with the molecular motor dynein and the multiprotein complex dynactin. The dynactin complex includes the subunits p150 and dynamitin, providing a link between the transported cargo, microtubules, and cytoplasmic dynein (reviewed in 288). Mutations in the p150 subunit (the *dctn1* gene) have been reported in both fALS and sALS cases (212, 213) in addition to atypical forms of the disease (Fig. 8) (243). A direct link between impaired dynactin/dynein function and motoneuron disease came from the finding that alterations on dynactin function results in motoneuron degeneration *in vivo* (163). Disease phenotypes observed by the expression of mutant dynactin in mice are characterized by drastic defects in vesicular trafficking, lysosomal proliferation, and distal degeneration in motoneurons (50, 318). Thus, the alteration in vesicle transport is a relevant factor in some forms of ALS, reinforcing the role of secretory pathway stress in the disease process.

V. Final Stop: Autophagy

A. Autophagy pathways

The proteasome and lysosomal pathways are the two main routes of protein clearance. Proteasomes predominantly degrade short-lived proteins, and their substrates need to be unfolded to pass through the narrow pore of the proteasome barrel (reviewed in 269). Increasing evidence indicates that the clearance of proteins through autophagy correlate with their propensity to aggregate (reviewed in 108, 269). Macroautophagy, here referred to as autophagy, is a highly conserved and regulated eukaryotic intracellular degradative process (Fig. 10) (171, 172, 204). Autophagy is regulated by a large family of genes, termed *atgs,* and it is involved in the catabolism of cytoplasmic components including damaged or superfluous organelles, toxic protein aggregates and intracellular pathogens. The autophagy process involves encapsulation of cargo into a double-membrane vesicle to form the autophagosome vesicle. Autophagosomes then fuse with lysosomes to form the autolysosomes where cargoes are degraded. In addition, hybrid intermediates called amphisomes are formed when autophagosomes fuse with the endosomes or MVEs before fusing with lysosomes (Fig. 10) (297, 321). ER stress and proteasome inhibition also trigger autophagy (62). For example, PERK/eIF2α and IRE1α/JNK signaling have been reported to initiate autophagy (158, 244, 273), acting as an important survival mechanism to cope with protein misfolding (49, 222). Unregulated or excessive autophagy is thought to be detrimental to cell survival due to uncontrolled organelle and macromolecular catabolism (172).

B. Regulation of autophagy

The mechanisms underlying the initial activation steps of autophagy are very complex and poorly understood. Atg proteins regulate different sequential steps in the autophagy process, starting with the formation of a protein kinase–autophagy regulatory complex and a lipid kinase–signaling complex that is involved in vesicle nucleation. Ubiquitin-like protein conjugation pathways are required for vesicle expansion and closure, and for the retrieval pathway for disassembling Atg protein complexes from mature autophagosomes. The core of the autophagic machinery is built around two ubiquitin-like conjugation systems (Fig. 10): ATG12 and microtubule-associated protein 1 light chain 3 (LC3). When ATG12 is activated, it forms a covalent bond with ATG5, which then forms a multimeric complex with other components, translocating to membranes of early autophagosomes. Additionally, LC3 is cleaved and then conjugated to phosphatidylethanolamine (PE), generating LC3-II. The unconjugated LC3-I remains in the cytosol while the

conjugated LC3-II form redistributes to the autophagosomal membrane after the formation of the active ATG12-ATG5 complex, leading to autophagosome membrane extension (172). Monitoring the generation of LC3-II is the gold standard to measure autophagy levels. p62 (also called sequestosome 1) serves as a bridge between poly-ubiquitination of proteins and the autophagy-degradative machinery (7, 289), operating as a shuttle factor for substrates of the proteasome to undergo autophagy-mediated degradation (18, 238).

One of the main autophagy regulatory pathways involves the participation of mTOR. mTOR signaling represses autophagy by the inhibition of a downstream class III phosphatidyl inositol 3-kinase (PI_3K) complex (Fig. 11). Activation of autophagy by nutrient deprivation involves the derepression of the mTOR kinase. An initial step for vesicle membrane nucleation is the activation of a complex containing VPS34, a class III PI_3K (111). VPS34 activation depends on the formation of a multiprotein complex including beclin-1 (the mammalian orthologue of ATG6). In addition, recent evidence suggests existence of a regulatory network where inositol-1,4,5-triphosphate receptor (IP_3R) mediates the initiation of autophagy through a protein complex with beclin-1 at the ER membrane, acting as a scaffold protein independent of its calcium channel activity (340). Autophagy is critical for the maintenance of neuronal homeostasis and contributes to basal elimination of misfolded proteins in the nervous system. Brain specific ablation of *atg5* and *atg7* leads to spontaneous neurodegeneration with pathological features closely resembling Alzheimer's disease and Parkinson's disease, consisting of neurological/motor dysfunction, accumulation of poly-ubiquitinated protein aggregates, neuronal loss, and premature death (104, 156).

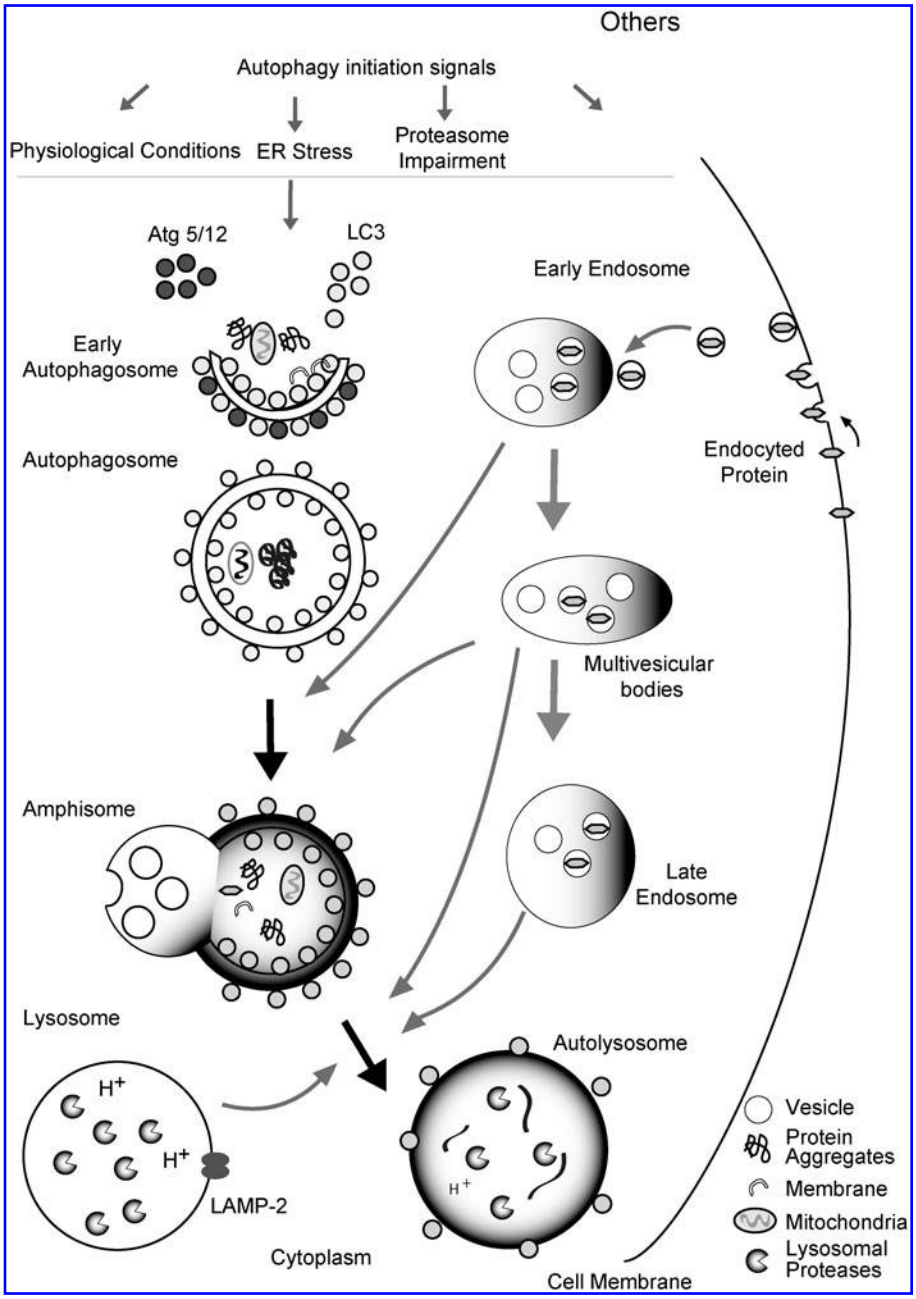

FIG. 10. Autophagy-related vesicles in eukaryotic cells. A variety of physiological and noxious signals (i.e., nutrient starvation, protein misfolding, and organelle damage) induce autophagy. The autophagy process begins with the nucleation of a protein complex to form the growing phagophore membrane. Then the vesicle expands and encapsulates sequestered materials including cytosol, organelles, and protein aggregates, forming the double membrane vesicle autophagosome. Autophagosomes then fuse with endocytic compartments including endosomes, multivesicular bodies, and lysosomes. Amphisomes are organelles containing both autophagosome and endosomal constituents. Amphisomes and autophagosomes mature by fusing with lysosomes, forming the autolysosome in which encapsuled cellular content is degraded by hydrolytic enzymes and proteases.

Recent studies suggest that upstream mTOR-independent pathways, previously undefined, modulate the core autophagy machinery. This pathway is becoming relevant for treatment of neurological conditions, and recent drug screenings have uncovered regulatory networks of this pathway (257, 277–279, 281). mTOR-independent autophagy is induced by agents that lower or deplete inositol or IP_3 levels such as lithium, valproate, and carbamazepine (Fig. 11) (278, 280). Inhibition of inositol monophosphatase (IMPase), an enzyme participating on inositol monophosphate turnover, triggers autophagy (278, 280). In addition, small sugars such as the disaccharide trehalose have been also shown to trigger mTOR-independent autophagy by an unknown mechanism, having therapeutic effects on models of Huntington's disease, Alzheimer's disease, and prion disorders, among other pathologies (15, 58, 251, 315).

Finally, a recent study added more complexity to the regulation of autophagy. Nishida and co-workers described the occurrence of ATG5- and ATG7-independent autophagy in cells undergoing stress triggered by DNA damage associated with the appearance of classical morphological and functional autophagy features (226). However, this form of autophagy was independent of LC3, but depends on the expression of beclin-1 and unc-51-like kinase 1 (the human ATG1 ortholog). Moreover, unlike conventional autophagy, autophagosomes were proposed to be generated by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes and not the ER (226). These results suggest the occurrence of autophagy through at least two different core pathways: an ATG5/ATG7-dependent conventional pathway and an ATG5/ATG7-independent pathway. It remains to be

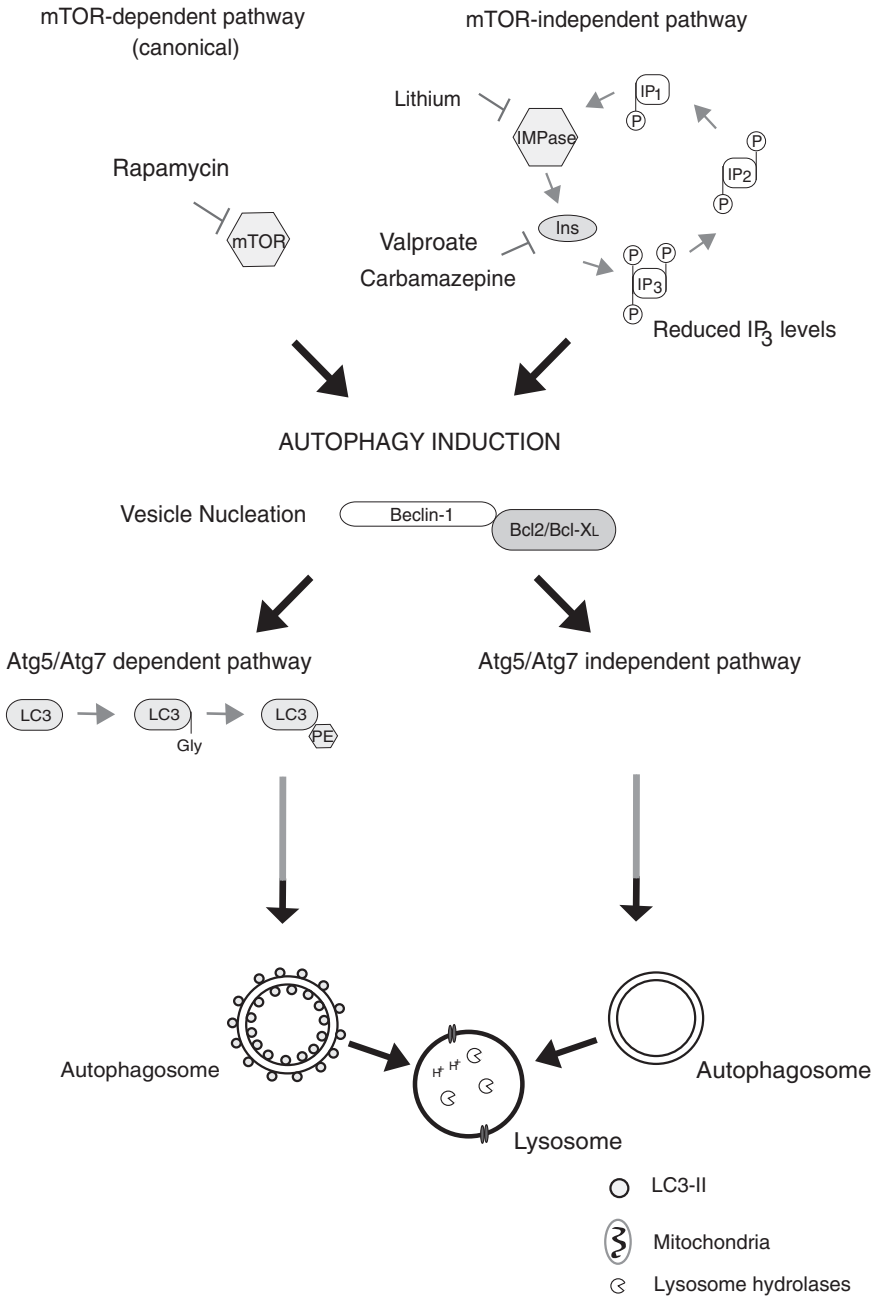

FIG. 11. General regulatory mechanisms of autophagy. Two main pathways are involved in autophagy activation: The mTOR-dependent and mTOR-independent pathways. The first occurs upon de-repression of mTOR Ser/Thr kinase (mammalian target of rapamycin) that is triggered by nutrient conditions or experimentally by rapamycin treatments. A second pathway is initiated by a decrease in global inositol phosphate (IP_3) levels. This pathway is modulated by IMPases, and can be induced by lithium, valproate or carbamazepine treatments. Recruitment of many protein complexes occurs during vesicle nucleation that involves the activation of the autophagy regulator Beclin-1 and PI3 kinases. BCL-2/BCL-X_L negatively regulates this nucleation step by binding to Beclin-1. Atg5 and Atg7 have been shown to be essential regulators of autophagy. The conjugation of phosphatidylethanolamine (PE) to LC3/Atg8 leads to the conversion of the soluble form of LC3 (named LC3-I) to the autophagosome-associated form (LC3-II). Autophagosomes mature by fusion with lysosomes, creating autolysosomes, in which the luminal content of the autophagic vacuoles is degraded. Alternatively, autophagy can proceed through a mechanism independent of atg5/atg7 and LC3, but dependent on Beclin-1 expression.

determined how beclin-1 differentially senses distinct autophagy stimuli (i.e., nutrient deprivation versus DNA damage) to engage these two mechanistically independent autophagy pathways.

C. Autophagy in fALS

Different studies in fALS models indicate that autophagy may operate as an active cellular response to clear out mutant SOD1 aggregates (Fig. 12). Kabuta and colleagues initially reported that activation of autophagy reduces mutant $SOD1^{G93A}$-mediated toxicity in a neuroblastoma cell line (137). Mutant SOD1 degradation was suppressed by treatments with PI_3K inhibitors, and by the administration of the mTOR inhibitor rapamycin. Inhibition of autophagy leads to a drastic accumulation of mutant SOD1 aggregates, enhancing its neurotoxicity (137). We have recently confirmed this data and further demonstrated a crucial role of ATG5 and beclin-1 in the degradation of mutant SOD1 oligomers in cellular models of fALS (121).

p62 has been observed in ubiquitin-positive inclusions in ALS (83, 203, 220, 239), in addition to a variety of neurodegenerative diseases such as Alzheimer's disease (7, 159), Huntington's disease (18, 216), and Parkinson's diseases (159, 221). It has been reported that p62 preferentially interacted with mutant SOD1 (83), facilitating its sequestration into inclusions (83, 84). p62 modulates the targeting of mutant SOD1 for autophagy- and proteasome-dependent degradation in both ubiquitin-dependent and -independent manners (Fig. 12) (84).

Studies in mutant SOD1 transgenic mice have provided a correlation between disease progression and autophagy induction. The levels of LC3-II are increased in $SOD1^{G93A}$ transgenic mice at the symptomatic stage (207). Moreover, phosphorylation of mTOR at Ser2448 was shown to be enhanced in the same experiments. Another group confirmed the presence of LC3-positive vesicles in fALS mouse models (175). We have also described the activation of autophagy in the spinal cord of $SOD1^{G86R}$ transgenic mice as monitored by the presence of LC3-positive vacuoles and the co-localization of SOD1 and LC3 into autophagosomes by electron microscopy and immunogold staining (121). This event correlated with the upregulation of beclin-1 in the same tissue. It has been also suggested, using skeletal muscle-specific mutant $SOD1^{G93A}$ transgenic mice, that autophagy may have a noxious role (64). The authors suggested that the occurrence of oxidative stress in the disease induces abnormal activation of FoxO3, NF-κB, and autophagic-related signaling events, resulting in muscular atrophy.

Finally, recent reports suggested a role of autophagy in the degradation of cytoplasmic aggregates of ubiquitinated TDP-43 (Fig. 4) (133, 334, 344). In addition, inhibition of mTOR or PI3K reduces the accumulation of TDP-43 C-terminal fragments and restores TDP-43 normal localization (25), whereas trehalose treatment decreased TDP-43 levels (344), suggesting a more general role of autophagy in different forms of ALS. Finally, expression of human TDP-43 protein in Drosophila motor neurons led to motor dysfunction and dramatic reduction of lifespan and the coexpression of ubiquilin-1 reduced TDP-43 expression. Ubiquilin-1 was previously identified as a TDP-43-interacting protein with suspected functions in autophagy and proteasome targeting. All these reports together suggest an interconnection between protein aggregation and autophagy in ALS, reflecting clear disturbances of proteostasis in the disease process.

D. A role of Fig4 mutations in autophagy and ALS

Mutations of the lipid phosphatase *Fig4* that regulate the generation of $PI(3,5)P_2$ are responsible for the recessive peripheral nerve disorder Charcot–Marie–Tooth disease type 4 (CMT4J). Interestingly, a nonsynonymous variant of *Fig4* was described in $\sim$1% of ALS patients (53). Mutations of *Fig4* resulted in a 50%–75% reduction in the levels of $PI(3,5)P_2$. *Fig4* mutations trigger progressive neurodegeneration and premature death in mice (54, 132, 365). PI(3,5)P2 is synthesized on endosomes and lysosomes in response to specific cargo molecules (65). Of note, mutant mice on *Fig4* or *vac14* (another component of the $PI(3,5)P_2$ regulatory pathway) depicted a clear accumulation LC3-II, p62 and LAMP-2 in neurons and astrocytes (Fig. 4) (72). Cytoplasmic protein inclusions containing p62 and ubiquinated proteins were present in brain regions that undergo degeneration. Unexpectedly, the majority of p62 accumulated in the brain was located within astrocytes. The authors indirectly suggested that *Fig4* mutations may lead to impaired autophagy, triggering neurodegeneration similar to the phenotype described for autophagy-deficient mice (104, 156).

E. Homeostatic balance between autophagy and the UPR in ALS

As mentioned in previous sections, a crosstalk between the UPR and autophagy has been described in many experimental systems, suggesting that both stress pathways are interconnected to decrease protein misfolding levels. For example, a drastic reduction in the generation of mutant SOD1 aggregates was observed in cells and mice where *xbp1* expression was inhibited. Using pharmacological and interfering RNA strategies (i.e., targeting *beclin-1* or *atg5*), we showed that upregulation of autophagy was responsible for the reduced accumulation of mutant SOD1 aggregates in XBP1 deficient cells (121). Through complementary assays, such as LC3-flux assays, measurements of lysosomal activity, electron microscopy (EM) studies, and pharmacological approaches, we demonstrated increased basal autophagy in *xbp1* knockdown cells without any additional autophagic stimuli. *In vivo*, *xbp1* deficiency in the nervous system resulted in significant increase in the lifespan of mutant SOD1 female mice associated with a marked increase of LC3-positive neurons and autophagosomes in spinal cord ventral horn. Interestingly, most LC3-positive cells were neurons and not glial cells, indicating a specific contribution of XBP1 deficiency to the enhancement of autophagy in neurons (121). Enhanced induction of beclin-1 was also observed in the spinal cord of *xbp1*-deficient mutant SOD1 strain. This study suggested a complex homeostatic rheostat where XBP1 expression modulates the basal activity of autophagy in the nervous system, providing protection against ALS potentially due to the elimination of abnormal protein aggregates (194) and maybe damaged organelles.

F. Autophagy in sALS

We recently reported an analysis of human postmortem samples from patients affected with sALS and fALS revealed

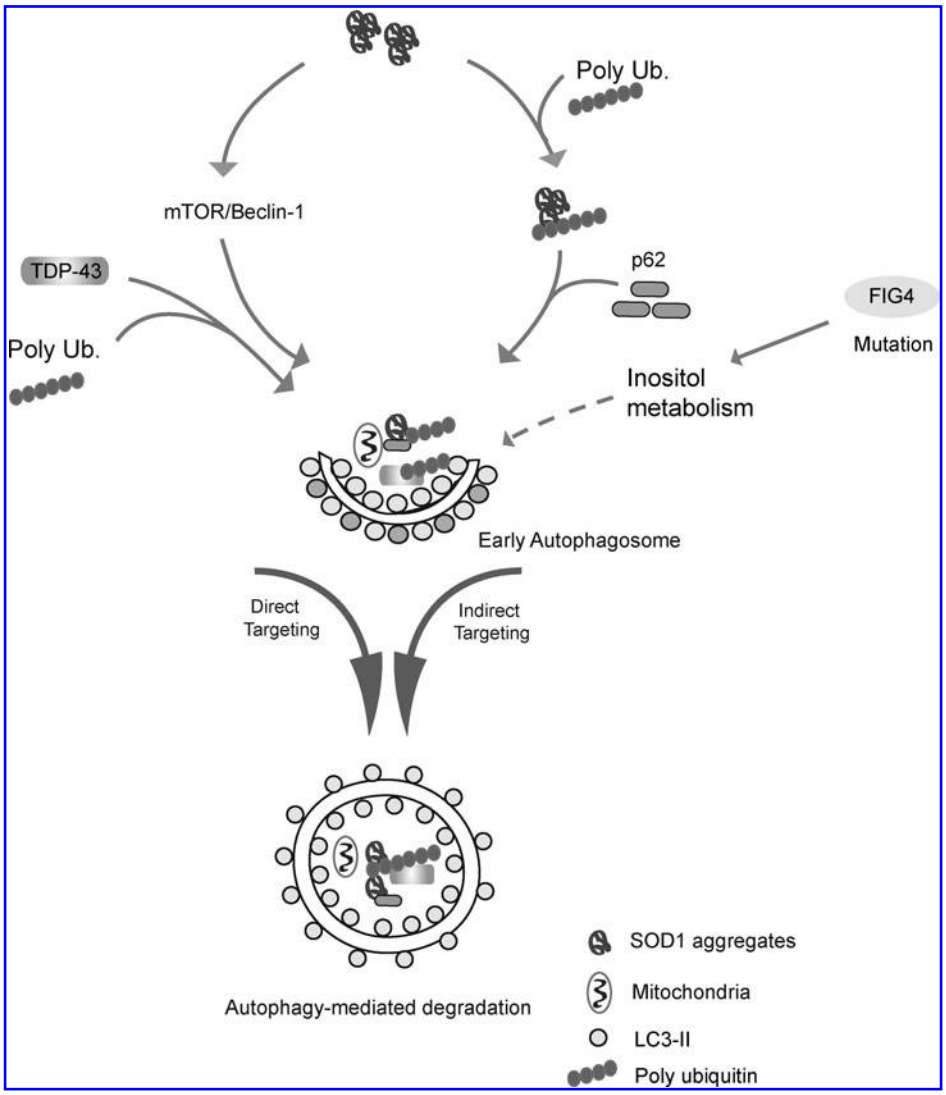

FIG. 12. Autophagy pathway in ALS. Intracellular aggregation and accumulation of mutant SOD1 occurs during ALS progression. p62 links the proteasome system to the autophagy pathway through the binding to ubiquitinated proteins (including SOD1) and their targeting to autophagosomes. The adaptor p62 protein delivers mutant SOD1 to autophagy possibly by a direct interaction. TDP-43 is also ubiquitinated and targeted to autophagy-mediated degradation. On the other hand, mutations in *fig4* interfere with inositol metabolism, possibly altering autophagy levels through an inhibitory mechanism.

a marked induction of LC3-II, ATG5-ATG12, and beclin-1 in several cases (121). Upregulation of beclin-1 closely correlated with the accumulation of ubiquitinated proteins and the upregulation of several ER chaperones in spinal cord tissue (121).

G. Autophagy as a therapeutic target to treat ALS?

Based on compelling data discussed in previous sections that suggest a key role of protein misfolding on sALS and different forms of fALS, therapeutic benefits of targeting autophagy are becoming more attractive for treatment of this pathology and other disorders related to abnormal protein aggregation (121, 193). Drugs such as lithium, carbamazepine, and sodium valproate, all which trigger mTOR-independent autophagy, are known to augment basal autophagy levels and enhance the clearance of autophagy substrates in cellular models (71, 197, 278, 280). In recent years, an intense debate has gone on regarding the use of lithium to treat patients with ALS. A small and preliminary human trial suggested protective effects of lithium treatment in ALS patients (77). Some groups have also reported positive effects in the treatment of animal models of ALS with lithium or in combination with other compounds such as valproate and riluzol (27, 77). However, others reported that lithium treatments exhibit a marginal effect on disease onset in fALS animal models (71, 86, 295). Surprisingly, Pizzasegola and co-workers actually observed acceleration in disease onset in mutant SOD1 mice treated with lithium, with impairment in motor functions and anticipation of time of death in two different mouse strains (245). Interestingly, a recent double-blind, placebo-controlled trial demostrated that combination with riluzole slows progression of ALS more than riluzole alone. The time-to-event endpoint and use of prespecified interim analyses enabled a clear result to be obtained rapidly. This design should be considered for future trials testing the therapeutic efficacy of drugs that are easily accessible to people with ALS (92).

Other known autophagy-inducing agents remain to be tested in the context of ALS. Interestingly, a recent report indicated that long-term *in vivo* treatment with rapamycin is well tolerated in mice and, remarkably, leads to extended lifespan (107). Similarly, deletion of *ribosomal S6 protein kinase 1* in mice, a component of the mTOR signaling pathway, lead to increased lifespan and resistance to age-related pathologies, including motor dysfunction and loss of insulin sensitivity

(291). Similarly, a recent report showed that inactivation of an mTor regulator in *Drosophila melanogaster*, Sestrin, lead to age-associated pathologies including triglyceride accumulation, mitochondrial dysfunction, muscle degeneration, and cardiac malfunction, which were prevented by pharmacological inhibition of mTOR (169). It remains to be determined if rapamycin treatment has any potential therapeutic effect for ALS.

VI. Conclusion

ALS is a fatal neurodegenerative disease with no effective treatment. Although ALS was first described in 1865 by Charcot, the primary mechanism mediating the selective degeneration and death of motoneurons still remains unclear, likely due to complexity of the disease at a molecular level. Most of clinical trials of drugs designed and validated in fALS mouse models have failed in human patients, possibly because they were often focused in targeting specific mutant SOD1-mediated effects. In addition, another important caveat is the experimental use of mouse models on a pure genetic background, a condition far from the scenario observed in nature. Since the same neurons and circuits are affected in both sALS and fALS, and both diseases are clinically very similar, we believe that the key strategy for designing new therapeutic strategies should be focused on identifying molecular events that are transversal to both sALS and fALS.

In this review, we have attempted to provide a systematic analysis to uncover common molecular features observed in both familial and sporadic ALS, dissecting the similarities and contradictions between recent studies. The data discussed here support an emerging concept where secretory pathway-related processes are crucial cellular events affected in ALS. It is possible to find alterations in ALS motoneurons at virtually every step of the secretory pathway. Because motoneurons are distinguished from other cells by their extreme asymmetry (up to one meter long), by a large volume (up to 5000 times bigger than a typical cell), and by active transport of diverse cellular components through the extended axon backward and forward, we predict that events affecting secretory pathway function will have devastating effects to neuromuscular function. As discussed here, the ER and GA are extremely affected at different levels in ALS, exhibiting diverse pathological events including GA fragmentation, protein misfolding, ER stress-mediated apoptosis, oxidative damage of mRNAs and proteins, ribosome detachment from the ER membrane, ERAD impairment, abnormal vesicular transport, altered autophagy, and accumulation of ubiquitinated protein inclusions.

Many studies using animal and cellular models of fALS triggered by distinct ALS-linked mutant genes indicate the occurrence of common pathological processes related to secretory pathway stress and protein misfolding. More importantly, recent studies have systematically evaluated the role of the UPR at diverse stages of the disease. Remarkably, ER stress was shown to occur very early during the presymptomatic phase of experimental fALS, even before denervation (284), suggesting that secretory pathway stress is one of the primary events on the disease process. Moreover, at the end stage of the disease, the occurrence of chronic ER stress has been linked to motoneuron loss. Based on the literature discussed in this review, the occurrence of ER stress emerges as a common consequence of alternating the function of the secretory pathway in ALS. Thus, many pathological events described in both sALS and fALS may converge into abnormal protein folding at the ER possible due to: (i) ERAD impairment, (ii) altered ER chaperone network, (iii) local oxidation of proteins at the ER (i.e., PDI dysfunction), (iv) disrupted vesicular trafficking, (v) altered cytoskeleton structure, (vi) GA network fragmentation, (vii) altered lysosomal/autophagic activity, among other pathogenic events. Taken together, all this evidence suggests that therapeutic strategies to alleviate secretory pathway/protein folding stress may have actual beneficial effects to treat ALS. Along this line, we envision different possibilities for therapeutic intervention. Among them, strategies to alleviate ER stress and recover proteostasis by increasing the folding capacity may be feasible using pharmacological strategies that could include treatments with chemical chaperones, activators of UPR signaling components, or gene therapy approaches to deliver key folding mediators. A second possibility may involve enhancing degradative/clearance pathways of misfolded proteins, such as ERAD and autophagy. This approach should alleviate the load of unfolded proteins at different secretory pathway subcompartments.

The predicted problem of most small molecules available to decrease protein misfolding is that they target generic cellular processes, and their use in humans may have pleiotropic effects for prolonged treatments. Exhaustive small molecule screenings in neuronal cultures are required to generate selective and efficient drugs to target specific events involved in secretory pathway stress. Because protein misfolding has also been suggested to be a molecular signature of diverse diseases including prion-related disorders, Huntington's disease, Parkinson's disease, Alzheimer's disease, among other diseases (Table 3), such drugs may benefit a wide range of affected patients (reviewed in 178, 193, 241, 269, 285). In summary, the

Table 3. ER Stress and Neurodegeneration

	ER Stress Markers in Cellular and Animal Models		*Human Disorder*
Disease	*In vitro*	*In vivo*	*(postmortem)*
Alzheimer's disease	+	+	+
Parkinson's disease	+	+	+
Amyotrophic lateral sclerosis	+	+	+
Prion disorders (TSEs)	+	+	+
Retroviral spongiform degeneration	+	+	n.d.
Huntington disease	+	+	+
Kennedy disease	+	n.d.	n.d.
Spinicerebellar ataxias	+	+	n.d.
Vanishing white matter disease	n.d.	n.d.	+
Brain ischemia/ brain trauma	+	+	n.d.
Niemann–Pick disease	+	n.d.	n.d.

The table summarizes evidence obtained from several animal and cellular models of neurodegeneration. Experimental validation for activation of the UPR in human patients affected with neurological diseases is also indicated from analysis of postmortem samples. For detail references, see the text in specialized reviews (193, 211, 263, 296, 313). n.d.: Not determined, in case of information still missing.

evidence discussed support the involvement of secretory pathway stress in both sporadic and most familial ALS variants, highlighting the notion that future therapeutic strategies to alleviate organelle damage and improve secretory pathway-related functions may lead to effective and transversal treatments for ALS and other protein misfolding disorders.

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Abbreviations Used

4-PBA = 4-phenylbutyrate
ALS = amyotrophic lateral sclerosis
ASK1 = apoptosis signal-regulating kinase 1
ATF4 = activating transcription factor 4
ATF6 = activating transcription factor 6
BAK = Bcl-2 homologous antagonist/killer
BAX = Bcl-2 associated X protein
BCL-2 = B cell lymphoma 2
BID = BH3 interacting domain death agonist
BIM = Bcl-2 interacting mediator of cell death
BiP = immunoglobulin binding protein
Cg = chromagranin
CHMP2B = charged multivesicular body protein 2B
CHOP = C/EBP homologous protein (see GADD153)
DERLIN = Der1-like domain
eIF2α = eukaryotic translation initiation factor 2α
ERAD = ER-associated degradation
ERK = extracellular signal-regulated kinase
ERp57 = ER protein 57 (also named GRP58)
fALS = familial ALS
FIG4 = lipid phosphatase FIG4
FUS = fused in sarcoma protein
GADD34 = growth arrest and DNA damage inducible protein 34
GADD45 = growth arrest and DNA damage inducible protein 45
GADD153 = growth arrest and DNA damage inducible protein 153 (also named CHOP)
GEFs = guanine-nucleotide-exchange factors
GRP78 = glucose-regulated protein 78
GRP94 = glucose-regulated protein 94
IRE1 = inositol requiring kinase 1
JNK = c-jun N-terminal kinase
LDCV = large-dense core vesicle
mTOR = mammalian target of rapamycin
NF-κB = nuclear factor-kappa B
NOXA = Latin for damage
PDI = protein disulfide isomerase
PERK = PRKR-like endoplasmic reticulum kinase
PUMA = p53-upregulated modulator of apoptosis
ROS = reactive oxygen species
sALS = sporadic ALS
SOD1 = superoxide dismutase 1
TDP-43 = tar DNA binding protein 43
TRAF2 = TNF receptor-associated factor 2
TUDCA = tauroursodexycholic acid
UPR = unfolded protein response
VAMP = vesicle-associated membrane protein
VAPB = vesicle-associated membrane protein/synaptobrevin-associated membrane protein B
XBP1 = X box-binding protein 1
XBP1s = X box-binding protein 1 spliced

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