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COMPREHENSIVE INVITED REVIEW

# Amyotrophic Lateral Sclerosis: New Insights into Underlying Molecular Mechanisms and Opportunities for Therapeutic Intervention

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**Abstract**

Recent years have witnessed a renewed interest in the pathogenic mechanisms of amyotrophic lateral sclerosis (ALS), a late-onset progressive degeneration of motor neurons. The discovery of new genes associated with the familial form of the disease, along with a deeper insight into pathways already described for this disease, has led scientists to reconsider previous postulates. While protein misfolding, mitochondrial dysfunction, oxidative damage, defective axonal transport, and excitotoxicity have not been dismissed, they need to be re-examined as contributors to the onset or progression of ALS in the light of the current knowledge that the mutations of proteins involved in RNA processing, apparently unrelated to the previous "old partners," are causative of the same phenotype. Thus, newly envisaged models and tools may offer unforeseen clues on the etiology of this disease and hopefully provide the key to treatment. *Antioxid. Redox Signal.* 17, 1277–1330.

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## I. Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron (MN) disease with a worldwide incidence of around 2.5 cases per 100,000 people per year, with a slightly higher incidence of disease in men, and a prevalence of 4–6 cases per 100,000 people. Its most typical feature is the degeneration of cortical, bulbar, and spinal MNs, except for those that control the bladder and the eye movement. This leads to generalized muscle weakness, leading to progressive paralysis until death caused by respiratory failure. The average age of onset is 50 years, and the typical survival time is 3–4 years after diagnosis, but this parameter is influenced by age at symptom onset, clinical presentation, age at respiratory dysfunction, and nutritional state of the patient (122, 290).

Approximately 10% of ALS cases are inherited (familial ALS [fALS]), with multiple autosomal dominant and recessive forms, but ALS is sporadic in the vast majority of patients (sALS). sALS and fALS share common clinical and neuropathological features, and ALS patients show some degree of heterogeneity as far as symptoms, age of onset, and disease duration are concerned.

The causes of sALS are still obscure. Various environmental risk factors have been suggested, such as consumption of food with high concentrations of the neurotoxic aminoacid β-methyl-amino-L-alanine (105); use of cholesterol-lowering drugs (201); intensive physical exercise (226), including football playing in the Italian professional leagues (91) and in amateur teams in England (581); and service in the U.S. Army (279). Strenuous exercise and military service in the Gulf War as risk factors for ALS may be linked to either intermittent occupational hypoxia (548) or head injury (85, 360, 466), but in a different study, significant elevation of risk in association with head trauma was only found within the first year after injury, and, thus, may likely be a consequence of initial ALS, causing a tendency to fall (537).

Cigarette smoking is also considered a risk factor for developing ALS (187, 466), and it is hypothesized that this could occur through lipid peroxidation *via* formaldehyde exposure. Environmental toxicants such as heavy metals (275), pesticides or herbicides (61, 67, 509), and other chemicals may be considered risk factors in relation to impaired detoxification in sALS patients (378, 379). In a systematic review of the literature on occupation as a potential determinant of ALS, "veterinarians and other health workers, athletes, hairdressers, power-production plant, electrical and military workers" were found to be candidate workers at risk for ALS, although further evaluation in well-designed studies is needed (508).

The current standard therapy provides support in breathing and also in feeding in advanced stages of the disease, and a number of pharmacological treatments for symptom relief, with only one drug, riluzole, specifically aim at slowing the progression of the disease. Riluzole is a molecule with multiple effects that inhibits the release of glutamate, blocks α-amino-3-hydroxy-5-methyl-4-isoxazole propinate (AMPA) receptors (36, 83), and has a mild antioxidant activity (300, 498). Riluzole administration has a positive effect on survival in a commonly employed ALS mouse model (217) but slows the disease in humans only slightly, prolonging survival by 3 months and not clearly improving the quality of life of patients (370).

Several other treatments are currently in various phases of clinical trials (http://clinicaltrials.gov/) and span from anti-inflammatories to neurotrophic, antioxidants, and anti-apoptotic molecules (560). The choice of drugs currently tested clearly reflects the general consensus that ALS is a multifactorial and a multi-systemic disease, with a number of intertwined mechanisms which produce alterations inside MNs and their neighboring nonmotoneuronal cells and that concur to the onset of symptoms and progression of the disease.

Due to the early discovery of mutations in the gene coding for the antioxidant enzyme Cu, Zn superoxide dismutase (SOD1) (448), since the past 17 years, much research has been

spent on models *in vitro* and *in vivo* based on the overexpression of the mutant forms of this protein. However, as detailed next, SOD1-linked ALS represents only one-fifth of ALS with a clear genetic origin and a minority of total ALS cases. Thus, it is clear that any findings on SOD1 models and patients need to be generalized. This concept is further supported by recent genetic evidence that fALS arises through mutation in proteins apparently unrelated to each other. Thus, it is clear that different pathways should converge to generate the same pathological phenotype, but the point of convergence is still not obvious. This review will summarize current knowledge on various pathways involved in ALS, with a focus on possible intersections.

## II. Genetics of ALS

### A. ALS loci

fALS can be inherited as either an autosomal dominant or an autosomal recessive trait. Linkage studies indicated that both types of inheritance are represented by more than one distinct genetic entity. Several loci have been identified by genetic analysis, and several have been assigned to specific genes (Table 1). Although in several cases the function of each mutant protein is not yet clearly identified or, even worse, too many different functions have been proposed, the evidence available suggests that fALS proteins are involved in a wide range of cellular processes, from antioxidant responses to axonal and vesicular transport, angiogenesis, and RNA processing.

1. ALS1–SOD1. About 20% of fALS patients carry mutations in the locus ALS1 on chromosome 21 that codes for SOD1 (EC 1.15.1.1).

After the first report in 1993 (448), about 140 different SOD1 mutations have been described in ALS families (http://alsod.iop.kcl.ac.uk/) mostly as acting dominantly except 2 that are linked to recessive inheritance. With a few exceptions clustered in exon 4 and 5 (eight frameshift deletions and five insertions, which lead to a premature truncation of the protein), all are point mutations distributed in all five exons of the gene and result in alteration of the residues scattered throughout the 153 amino-acid protein sequence. Since some mutations affect the metal-binding residues at the active site, while others affect the proper folding or stability of the homodimer, the biophysical and biochemical properties of ALS-associated mutant SOD1 proteins are quite heterogeneous (474). Many mutant SOD1s retain their superoxide scavenging activity *in vitro* and *in vivo*, thus indicating that SOD1 mutations are generally not related to decreased SOD activity. Moreover, knockout mice for SOD1 do not show an ALS-like phenotype, although these mice have axonal degeneration, increased mitochondria-dependent oxidative stress (172), and an accelerated loss of muscle mass and function with age (301, 555). Therefore, it is widely accepted that mutations in SOD1 confer a toxic additional function to the protein, which may be linked to the intracellular localization of the enzyme, as described in detail next.

2. ALS2–Alsin. Mutations in the locus ALS2 were initially found in two families (220, 597) and were found to be associated not only with different forms of rare autosomal-recessive, juvenile MN disease (152, 153, 220, 302, 597), but also with other conditions such as primary lateral sclerosis (414).

Except for 2 missense mutations, all of the 13 different *ALS2* mutations described so far lead to a premature stop codon. The *ALS2* gene is composed of 34 exons and through alternative splicing, it encodes two forms of the protein alsin that differ by size, respectively a 1657-residue, long form that contains three different guanine-nucleotide-exchange factor-like domains, which may play a role in the etiology of the disease, and a 396-residue, short form (78). The mutations affecting both the long and short form of the protein seem to result in the juvenile ALS phenotype, while those affecting the long form only may be responsible for milder phenotypes (juvenile primary lateral sclerosis or infantile-onset ascending hereditary spastic paraparesis) (523).

Alsin is expressed in various tissues and cells, including neurons throughout the brain and spinal cord; its localization onto the cytosolic face of endosomal membranes and its function as a guanine nucleotide exchange factor for Rab5 and other small GTPases (411, 528) suggest its role in endosomal

Table 1. Familial Amyotrophic Lateral Sclerosis Subtypes and Their Genetic Determinants

| *ALS-type* | *Onset* | *Inheritance* | *Locus* | *Gene* | *Protein* | *References* |
|---|---|---|---|---|---|---|
| ALS 1 | A | AD | 21q22.1 | *SOD1* | Cu/Zn superoxide dismutase | (478) |
| ALS 2 | J | AR | 2q33-35 | *ALS2* | Alsin | (233) |
| ALS 3 | A | AD | 18q21 | Unknown | Unknown | (223) |
| ALS 4 | J | AD | 9q34 | *SETX* | Senataxin | (88) |
| ALS 5 | J | AR | 15q15-21 | *SPG11* | Spatacsin | (234) |
| ALS 6 | A | AD | 16p11.2 | *FUS* | Fused in sarcoma | (1, 451, 456) |
| ALS 7 | A | AD | 20p13 | Unknown | Unknown | (456) |
| ALS 8 | A | AD | 20q13.33 | *VAPB* | VAMP-associated protein B | (398) |
| ALS 9 | A | AD | 14q11.2 | *ANG* | Angiogenin | (208) |
| ALS 10 | A | AD | 1q36 | *TARDBP* | TAR-DNA-binding protein | (198, 272, 305, 453, 490) |
| ALS 11 | A | AD | 6q21 | *FIG4* | PI(3,5)P(2)5-phosphatase | (94) |
| ALS 12 | A | AD | 10p15-p14 | *OPTN* | Optineurin | (354) |

The table summarizes the different fALS subtypes, the onset and the type of inheritance. Chromosome location, the gene, and the protein associated to each subtype and the references reporting the association between each locus and ALS are shown.

A, adult; AD, autosomal dominant; AR, autosomal recessive; fALS, familial amyotrophic lateral sclerosis; J, juvenile; TAR, trans active response; VAMP, vesicle-associated membrane protein.

motility and degradation, vescicular trafficking, and cytoskeletal organization (133, 312), but studies *in vitro* and *in vivo* support its role in AMPA receptor trafficking as well (311).

Surprisingly, the loss of alsin has no drastic effect on the survival or function of MNs in mice (68, 69). Several lines of knockout mice for alsin were generated, and all appear to be viable, fertile, with no signs of hind limb weakness, and with a lifespan similar to that observed in wild-type littermates. Alsin−/− mice have no loss of upper or lower MNs, but show a distal axonopathy, which suggests that alsin plays an important role in maintaining the integrity of the corticospinal axons (132). This may cause the observed impaired motor performance (133, 593). Alsin-deficient mice also exhibit a decrease in cerebellar purkinje cells and a progressive enhancement of astrocytic, microglial, and macrophagic staining in the brain and spinal cord (219).

3. ALS4–SETX. Recessive mutations in the senataxin (SETX) gene cause ataxia-oculomotor apraxia type 2, a condition characterized by cerebellar ataxia, oculomotor apraxia, peripheral neuropathy, and immunodeficiency (380); while dominant mutations in this gene cause a rare, slowly progressing, juvenile form of ALS in four families (88). SETX encodes a 302.8-kD protein that has several postulated functions. Based on protein homology, SETX was predicted to be a putative DNA/RNA helicase. However, in a recent study, the effect of the modulation of wild-type and mutant SETX on neuronal differentiation was investigated in primary hippocampal neurons. The overexpression of wild-type SETX is required and sufficient to trigger neuritogenesis, and it protects cells from apoptosis during differentiation; while the overexpression of the dominant mutant forms does not affect the regular differentiation process, supporting the concept that haploinsufficiency is the pathogenic mechanism implicated in ALS4 (552). Several other SETX protein functions have been proposed as being responsible for ALS. The role of SETX has been postulated in DNA damage repair and telomere stability (121), either in the regulation of transcription and RNA processing (506) or in the response to DNA damage after oxidative stress (505), although single amino-acid deletions in SETX sensitize cells to death induced by different agents, while the whole protein deletion does not (7).

4. ALS5–SPG11. Mutations in the 40-exon gene coding for spatacsin (SPG11) have been described as being the single most common cause of autosomal-recessive hereditary spastic paraplegia with thin corpus callosum (495) and some form of juvenile parkinsonism (13). In a recent investigation conducted on 25 families with autosomal-recessive juvenile ALS and long-term survival, 10 families from Italy, Brazil, Canada, Japan, and Turkey were found to carry mutations in the same gene (409). The association of these variant spatacsins and ALS is supported by their co-segregation with the disease in all pedigrees, absence in controls, and with some (but not all) features of ALS neuropathology, such as a marked loss of anterior horn large motoneurons and of large myelinated fibres in a single patient.

Spatacsin is a 2443-amino-acid protein that is ubiquitously expressed, with the highest expression in the liver, followed by the ovary and the brain (391). In the nervous system, its expression is most prominent in the cerebellum, cerebral cortex, hippocampus, and pineal gland (495).

Not much is known about its function; sequence analysis shows that this protein includes a leucine zipper, a coiled-coil domain, and four putative transmembrane domains, suggesting that the protein may be a receptor or a transporter. However, when a green fluorescent protein (GFP)-spatacsin fusion protein is overexpressed in the COS-7 cells, it localizes in the cytosol, perinuclearly and also in the nuclei (495). The initial suggestion that spatacsin is involved in axonal transport (229) is supported by a study where a morpholino antisense oligonucleotide injection was used in zebrafish embryos; in this model, a decrease in spatacsin causes several developmental defects, including perturbation of axon formation and neuronal differentiation (489). However, spatacsin may have other functions besides a role in development, as three alternative transcripts are detected in the adult human brain (495).

5. ALS6–FUS. Mutations linked to classical ALS also occur in the gene encoding for the fused in sarcoma/translocated in liposarcoma (FUS/TLS) and account for about 5% of fALS cases and rare sporadic cases. To date, 47 mutations have been described in patients: they are missense mutations, truncations (R495X), deletions, or in-frame insertions (310).

Most of the FUS ALS-linked mutations are clustered at the C-terminal domain of the protein and, in particular, in the nuclear localization signal (NLS). As described in greater detail next, the redistribution of the protein from the nucleus to the cytoplasm is related to the onset and progression of the disease, and the mutations that affect amino-acid residues responsible for the binding to transportin receptors (thus affecting the nuclear import of the protein) result in a more severe disease progression (140).

Of note, missense variants in TAF15, an RNA-binding protein belonging to the same protein family as FUS, have been recently identified in patients with ALS (103). Similar to FUS, these variants accumulate as cytoplasmic foci when expressed in primary cultures of spinal cord neurons and induce neurodegeneration when expressed in *Drosophila*. However, the contribution of TAF15 to ALS pathology still needs to be determined.

6. ALS8–VAPB. ALS8, an atypical, early-onset, slowly progressive form of ALS, is linked to a dominant missense mutation in the vesicle-associated membrane protein (VAMP)-associated protein (VAP) B. VAPB mutations were first found in a Brazilian family and later in patients from Europe (86, 398). However, several studies failed to identify *VAPB* mutations in cohorts of patients with ALS (99, 297, 315), suggesting that mutations in the *VAPB* could be rare in ALS.

VAPB is a type II integral membrane protein that plays a role in vesicle trafficking in the endoplasmic reticulum (ER)-to-Golgi transport, in axonal transport of membrane components, and in the unfolded protein response (UPR), a process that suppresses the accumulation of unfolded proteins in the ER (353). Mutations in this gene have also been associated with other motoneuronal diseases, such as late-onset spinal muscular atrophy (SMA) and late-onset atypical ALS with slow progression (398).

It is interesting to note that the structural homolog of human VAPB in *Drosophila* regulates synaptic remodeling by affecting the size and number of boutons at neuromuscular junctions (NMJs). The transgenic expression of mutant VAPB in neurons of flies recapitulates major hallmarks of human

diseases, including locomotion defects, neuronal death, mislocalization of the protein, and aggregate formation (77). In a similar *Drosophila* model, mutant VAPB seems to act in a dominant negative fashion through the aggregation and recruitment of wild-type VAP into aggregates, and through the interference with signaling pathways at the NMJ (439).

In a recent elegant article using the induced pluripotent stem cells (iPSCs) technology, Mitne-Neto and co-workers found that VAPB protein levels are reduced in MNs derived from an ALS8 patient (375), but, in contrast to the *Drosophila* overexpression systems just mentioned, cytoplasmic aggregates were not identified. These results suggest that optimal levels of VAPB may play a central role in the pathogenesis of ALS8, in agreement with the observed reduction of VAPB in sALS.

7. ALS9–ANG. Angiogenin (ANG) is a hypoxia-regulated gene coding for a 123-residue, 14.1 kDa member of the pancreatic ribonuclease A superfamily. Single-nucleotide polymorphisms in the ANG gene have been first associated with ALS in the Irish and Scottish populations (208, 209); later studies reported ANG mutations in familial and "sporadic" ALS patients from the United States (588), Germany (162), Italy (98, 191), France (418), The Netherlands (545), and Sweden, but not in Poland (361), accounting for 2.3% of fALS and for 1.0% of sALS cases (523).

ANG is not only highly expressed in the nucleus and cytoplasm of spinal cord ventral horn neurons of both human fetal and adult tissue, but is also secreted in the extracellular matrix and interstitial tissue. Its RNase activity is important for the angiogenic activity in many tissues, and *in vitro* functional expression studies showed that the mutant protein loses most of its ribonucleolytic activity and angiogenic function, and is unable to translocate from the cytoplasm to the nucleus (588).

ANG may also play a role in neuronal differentiation, as in a cell-culture model of neuro-ectodermal differentiation, treatment with ALS-associated ANG variants hinders the ability of the neurites to extend and make contacts with neighboring neuritis and has a cytotoxic rather than a protective effect (500). ANG not only modulates the activity of vascular endothelial growth factor (VEGF), a cytokine that controls new blood vessel growth, but also supports the growth and survival of neurons. Despite some controversial results, VEGF is considered a putative modifier of ALS, at least in some populations (314), and the expression of VEGF is reduced in anterior horn cells and in the cerebral spinal fluid (CSF) from patients with ALS (60, 269). More recently, a novel role has been proposed for ANG in the response to stress. Knock-down of ANG, but not related ribonucleases, inhibits stress-induced production of tiRNA, which are tRNA-derived stress-induced fragments (594) that cause translational repression, inhibition of protein synthesis, and activation of a cytoprotective stress response program which is mediated by the formation of stress granules (SGs) (151, 256). Thus, ANG may belong to the group of ALS genes that are involved in RNA metabolism.

8. ALS10–TDP43. In 1995, trans active response (TAR)-DNA binding protein (TARDBP or TDP-43) was first identified as a 43-kDa cellular factor binding TAR regulatory sequence of LTR in the HIV-1 virus genome (hence its name, TAR DNA-binding protein) (412). The gene consists of six exons and encodes for a 414-amino-acid-long, nuclear protein belonging to the family of heterogeneous ribonucleoproteins (hnRNPs). More than 40 mutations have been found in ALS patients, accounting for about 5% of fALS cases (116).

With regard to several other genes, TDP-43 is also associated with a different neurological disease, namely frontotemporal dementia (FTD). FTD and ALS were once considered entirely separated pathological entities, because dementia is reported to be an uncommon symptom in ALS, and only a few patients with FTD eventually develop ALS. The discovery of three other common players in both the diseases, namely valosin-containing protein (VCP) (578), charged multivesicular protein 2B (CHMP2B) (104), and C9ORF72 (126, 442) (see next), has led to a rethinking of the previous classification and a consideration of "pure" FTD and ALS as the extremes in a continuum of disease (345).

9. ALS11–FIG4. Mutations in the phosphatidylinositol 3,5-bisphosphate 5-phosphatase (FIG4 or Sac3) gene are associated with recessive Charcot-Marie-Tooth disease type-4J (CMT4J), a disease clinically resembling MN disease and characterized by impaired trafficking of intracellular organelles (95). In a single study conducted on 473 ALS patients, mutations in FIG4 were found in four fALS and five sALS patients (94). Since all ALS-linked mutations reported are missense or nonsense, that is, affect a splicing site or cause a shift in translation initiation, they are predicted to cause the loss of functional protein.

FIG4 is a phosphatase that dephosphorylates the signaling lipid, phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2)]. It interacts with FAB1 (fALS) and VAC14 (voltage-dependent anion channel [VDAC]) in a protein complex that regulates the overall concentration of PI(3,5)P2 (142, 143, 452). Since the content of PI(3,5)P2 in endosomal membranes changes dynamically with fission and fusion events that generate or absorb intracellular transport vesicles, the regulation of PI(3,5)P2 may be critical for the survival of neural cells. Indeed, mice lacking VAC14 exhibit massive neurodegeneration, particularly in the midbrain and in peripheral sensory neurons. Cell bodies of affected neurons are vacuolated, and membrane-trafficking pathways are defective (608), a phenotype closely resembling that of mice lacking FIG4 (95), where early neurodegeneration in sensory and autonomic ganglia is followed by the loss of neurons with reduced numbers of large-diameter myelinated axons and slowed nerve conduction velocity. Mice with mutations in both FIG4 and VAC14 show the accumulation of microtubule-associated protein1 light chain 3 (LC3-II), p62, and LAMP-2 (lysosomal-associated membrane protein 2) in neurons and astrocytes and the formation of cytoplasmic inclusion bodies containing p62 and ubiquinated proteins in degenerating regions of the brain (161), thus supporting the role of PI(3,5)P2 and impaired autophagy in this kind of neurodegeneration.

10. ALS12–OPTN. Mutations in the gene coding for optineurin (OPTN) have been first described in patients with a retinal degeneration called primary open-angle glaucoma (POAG) (444). More recently, three types of OPTN mutations, different from those found in POAG, have been described in Japanese ALS patients with a heterogeneous course of disease (354). An analysis of Japanese cohorts revealed OPTN

mutations in about 0.25% of sALS and 3.5% of fALS patients (354, 503), and relatively high frequencies of OPTN mutations were found in a cohort of 247 fALS and sALS Italian patients (1.2% and 3.5%, respectively) (127). However, other studies questioned the relevance of OPTN mutations in fALS and sALS (368, 543).

Wild-type OPTN is a 557-amino-acid protein that is ubiquitously expressed in nonocular and ocular tissues such as the heart, the brain, the retina, trabecular meshwork, and nonpigmented ciliary epithelium in mice (444, 445). It is mostly localized in granules associated with the Golgi apparatus; interacts with huntingtin (156), transcription factor IIIA (381), and Rab8 (227); and may form complexes with Rab8, myosin VI, and transferrin receptor (599); thus, OPTN is supposed to be involved in signal transduction, gene expression, and, most importantly, in vesicular trafficking (51).

OPTN also acts as a negative regulator of nuclear factor kappa B (NF-$\kappa$B) in a negative feedback loop in which tumor necrosis factor $\alpha$-induced NF-$\kappa$B activity mediates the expression of OPTN, which itself functions as a negative regulator of NF-$\kappa$B (501). In transfected NSC-34 cells, the ALS mutant OPTN loses the ability to inhibit the activation of NF-$\kappa$B, and one mutation has a cytoplasmic distribution which is different from that of the wild-type or a POAG mutation (354). The same authors reported that TDP-43- or SOD1-positive inclusions of sporadic and SOD1 cases of ALS are also immunolabeled by antibodies against OPTN mutations, but in a larger later study, it was concluded that OPTN-positive inclusions are uncommon and detected in only one-third of TDP-43-positive sALS spinal cord and frontotemporal lobar degeneration (FTLD)-TDP (241); furthermore, large OPTN inclusions are detected in neither fALS with SOD-1 and FUS mutations, nor FTLD-FUS cases, and, thus, OPTN inclusions seem not to play a central role in the pathogenesis of ALS in general. In other studies, OPTN was found to co-localize with FUS in basophilic inclusions seen in postmortem samples of ALS patients with FUS mutations (255), and skeinlike inclusions immunoreactive for OPTN were found in all the sALS and fALS cases without SOD1 mutations, but neither in the cases with SOD1 mutations nor in two lines of transgenic mice overexpressing the mutant SOD1 (128). Thus, OPTN seems to associate with each of three major ALS-related proteins, that is, TDP-43, SOD1, and FUS (Table 2).

OPTN is itself an aggregation-prone protein (599); in a normal condition, the turnover of wild-type OPTN in neuronal cells involves mainly the ubiquitin-proteasome pathway, but when the protein is up-regulated or mutated, autophagy is activated to remove excess, aggregated OPTN (476).

11. Other genes. Besides the one just described and already classified as falling into ALS loci, several other genes have been reported as being associated with rare or atypical forms of ALS, or also associated with other neurological conditions such as FTD.

Sabatelli and co-workers looked at 245 sALS patients and 450 controls for sequence variants in the genes coding for subunits of the neuronal nicotinic acetylcholine receptors (nAChRs) that are a part of the glutamatergic pathway (455). Neuronal nAChRs are pentameric ligand-gated channels, consisting of different combinations of $\alpha$2–10 and $\beta$2–4 subunits that are widely expressed in several regions of the central and peripheral nervous system, including the spinal cord (286). Several missense variants were found with a significantly higher frequency in patients than in controls (6.1% *vs.* 1.3%), which are located in the regions encoding the intracellular loop of AChRs subunits. A detailed investigation by electrophysiology of the nAChRs formed using either wild-type or one of four distinct $\alpha$-subunit mutations, together with a common $\beta$4 subunit, showed an alteration of several functional properties of the receptor such as modified affinity for nicotine, reduced use-dependent rundown of nicotine-activated currents, and reduced desensitization, leading to sustained intracellular $Ca^{2+}$ concentration (455). Some of these alterations have been confirmed in a study using the expression of nAChRs subunits in cultured mouse cortical neurons (446).

This finding is in line with the hypothesis that increased MN vulnerability to glutamate-induced excitotoxicity is one of the leading mechanisms in sALS, and that the accumulation of rare, mildly deleterious mutations may be an important contributor to complex diseases, including the sALS. Whether mutations in nAChR subunits constitute a real liability factor for ALS, however, needs further confirmation in the light of previous reports that mutations in the genes coding for subunits of the same family are responsible for the other rare neuromuscular disorders, that is, familial nocturnal frontal lobe epilepsy, congenital myasthenia syndromes, and arthrogryposis multiplex congenita-type Escobar (494).

Similar considerations hold true, and further confirmation is also needed for a number of other recently reported genetic risk factors, such as variants of 5,10-methylenetetrahydrofolate reductase, a protein involved in neurotoxic

Table 2. Contents of Aggregates in Different Forms of Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration

| | *ALS-SOD1* | *ALS-TDP* | *ALS-FUS* | *ALS-UBQLN* | *FTLD-TDP* | *FTLD-FUS* | *a FTLD-U* |
|---|---|---|---|---|---|---|---|
| SOD1 | + | − | − | − | n.r. | n.r. | n.r. |
| FUS/TLS | − | n.r. | + | + | n.r | + | + |
| TDP-43 | − | + | − | + | + | − | − |
| Ubiquilin 2 | + | + | n.r. | + | + | n.r. | n.r. |
| Optineurin | ± | + | + | + | + | n.r. | n.r. |
| Ubiquitin | + | + | + | + | + | − | + |

ALS-SOD1, ALS due to *SOD1* mutations; ALS-TDP, ALS due to *TARDBP* mutations; ALS-FUS, ALS due to *FUS/TLS* mutations; ALS-UBQLN2, ALS due to *UBQLN2* mutations; FTLD-TDP, also known as FTLD-U, frontotemporal lobar degeneration with tau-negative, ubiquitin- and TDP-positive inclusions; aFTLD-U, atypical FTLD-U; FTLD-FUS, frontotemporal lobar degeneration with fused in sarcoma inclusions; +/−, present/absent; n.r., not reported.

homocysteine metabolism (304) or paraoxonases, a group of protein with a role in the prevention of lipid oxidation and in the detoxification of organophosphates (522).

Mutations in CHMP2B were found not only in families with autosomal-dominant FTD (481), but also in a population from the North of England in ~1% of cases with ALS and in 10% of those with lower MN-predominant ALS (104). Cox and co-workers also demonstrated by microarray analysis that MNs from CHMP2B cases, compared with controls, show a distinct gene expression pattern and that transfection of mutant CHMP2B into HEK-293 and COS-7 cells causes the appearance of large cytoplasmic vacuoles, aberrant lysosomal localization, and impairment of autophagy. CHMP2B is expressed in all major areas of the human brain (481) and belongs to the chromatin-modifying protein/CHMP family. The function of this protein in human beings is not well known, but its yeast ortholog is a component of the endosomal sorting complex required for transport III, a complex involved in the degradation of surface receptor proteins and the formation of endocytic multivesicular bodies, which plays a critical role in the trafficking of proteins between the plasma membrane, trans-Golgi network, and vacuoles/lysosomes (25, 26).

Interestingly, two other ALS genes, *ALS2* and *ALS8*, seem to be involved in disruption of the processes of endocytosis and vesicle trafficking, and this may also be the case for VCP, which has been found to be mutated by exome sequencing in an Italian family with autosomal dominantly inherited ALS (265). Again, such mutations do not seem to be uniquely associated with ALS, as mutations in VCP have previously been identified in families with inclusion body myopathy, Paget disease, and FTD (578). VCP is a 100-kD ubiquitously expressed multifunctional protein that is complexed with clathrin and is a member of the AAA+ (ATPase associated with various activities) protein family. It has been implicated in multiple cellular functions ranging from membrane trafficking and organelle biogenesis to maturation of ubiquitin-containing autophagosomes (267).

A single report links a mutation R199W in the D-amino-acid oxidase gene (DAO) to classical adult-onset fALS in a three-generation fALS kindred (373).

DAO is a 39.4-kDa homodimeric, peroxisomal FAD-dependent oxidase that catalyzes the oxidative deamination of D-amino acids to their corresponding ketoacids. In the central nervous system (CNS), DAO is expressed in both neuronal and glial cells (382) and enriched in the brainstem and the spinal cord (373), a fact that may be relevant to its function, as D-amino acids are synthesized in the CNS and may serve essential functions as neurotransmitters (484).

The residue Arg199 is highly conserved through evolution and lies close to the FAD binding site and between residuesTyr228 and His307, which play a key role in enzyme activity (526). Thus, it is not surprising that the effect of the R199W mutation seems to markedly reduce enzyme activity despite normal levels of mRNA expression (373). Since heterodimers can be formed between wild-type and R199W DAO, a dominant negative effect may take place, further inactivating the wild-type enzyme.

That mutations in DAO may be causative of neurodegeneration in general, and ALS specifically, is suggested by the observation that both neuronal cells lines and primary MN cultures expressing R199W DAO showed decreased viability and increased cytosolic ubiquitinated aggregates compared with cells expressing the wild-type protein (373). Furthermore, mice genetically lacking DAO have several neurological alterations, including enhanced hippocampal long-term potentiation, improved performance in spatial learning tests (346), and reduced locomotor activity (11); while in the most common SOD1 mouse model of ALS (G93A-SOD1 mice), the level of D-serine is increased, in analogy to what is observed in the spinal cord in cases of sALS (373). According to the first genetic study, mutations in DAO seem to account for only a 0.3% of familial cases, and a second study on 162 fALS patients revealed a rare polymorphism but failed to reveal further DAO-linked mutations (369). Thus, the pathogenicity of the R199W mutation still awaits further confirmation.

In another single recent report (129), mutations in UBQLN2, an intronless gene on the X chromosome, have been identified in a five-generation family with ALS, including 19 affected individuals. The disease is transmitted in a dominant fashion with reduced penetrance in women and ranges from classical ALS to ALS/dementia. Skein-like inclusions positive for ubiquilin 2 (the protein encoded by UBQLN2) are found in tissues from ALS patients, which are also immunoreactive with antibodies to ubiquitin, p62, TDP-43, FUS/TLS, and OPTN, but not to SOD1.

Furthermore, ubiquilin 2 was found to be a common component in the skein-like inclusions in many other forms of ALS (sALS, fALS without mutations in SOD1, TDP43, and FUS/TLS, ALS with dementia, fALS with SOD1 mutations, and fALS with a G298S mutation in TDP43; Table 2). In a neuronal cell-culture model (co-transfected Neuro-2a cells), both wild-type and mutant ubiquilin 2 were mostly distributed in the cytosol and formed inclusions that, depending on the level of expression of the two proteins, may colocalize with the C-term of TDP-43 (129).

Ubiquilin 2 is a member of the ubiquilin protein family which is characterized by the presence of an N-terminal ubiquitin-like domain that binds to subunits of the proteasome, and a C-terminal ubiquitin-associated domain that binds to polyubiquitin chains on proteins marked for degradation by the proteasome. Additionally, ubiquilin 2 has a repeat region, unique in the family, containing 12 PXX tandem repeats, and all five ALS-linked mutations identified involve proline residues in this short repeat region.

The exact function of ubiquilin 2 is not well understood. However, functional analysis with a reporter substrate showed that mutations in UBQLN2 lead to an impairment of protein degradation through the UPR in transiently transfected Neuro-2a cells and SH-SY5Y cells. Interestingly, a previous work has demonstrated that another member of the ubiquilin family, ubiquilin 1, plays a role in Alzheimer's disease and interacts with presenilins 1 and 2 (218) and TDP-43 (295).

Juvenile ALS with an autosomal recessive pattern of inheritance, found in a single Arabian family, may also be caused by mutations in the transmembrane domain of the Sigma-1 receptor (Sig-1R) (9). Sig-1R is an ER chaperone that binds a wide range of ligands and is involved in ion channel modulation and inositol 1,3,5-triphosphate receptors. Sig-1R is ubiquitously expressed and enriched in MNs of the brainstem and the spinal cord (9). The expression of Sig-1Rs is thought to be neuroprotective, and Sig-1R knockout mice have motor deficiency (357). The mutant protein found in ALS

Table 3. Genome-Wide Association Studies in Amyotrophic Lateral Sclerosis

| *Locus* | *Protein* | *No. of patients* | *References* |
|---|---|---|---|
| 1p32.1 | FLJ10986 | 386 | (144) |
| 12p11 | 1,4,5-triphosphate receptor 2 gene | 876 | (546) |
| 7q36.2 | DPP6 | 958 | (111) |
| 17q21 | Progranulin | 538 | (482) |
| 19p13.11 | UNC13A | 2323 | (547) |
| 8p21.1 | Elongator protein 3 homolog | 781 | (480) |
| 1q24.2 | Kinesin associated protein 3 | 1821 | (316) |
| 15q11.2 | Nonimprinted in Prader-Willi/Angelman syndrome 1 | 4434 | (47) |
| 9p21 | C9ORF72 | 442 | (309) |

GWAS have been very successful in identifying common genetic variations associated to numerous complex diseases. This kind of approach has also been used in the case of ALS with the aim of identifying possible single-nucleotide polymorphisms that are significantly associated with an increased risk of developing ALS. Up-to-date, GWAS have allowed the identification of a small number of ALS loci, possibly because this disease is a phenotypically variable syndrome and a genetically heterogeneous disease, with multiple gene products involved in motor neuron degeneration.

ELP3, elongator protein 3 homolog; GWAS, genome-wide associations studies; ITPR2, 1,4,5-triphosphate receptor 2 gene; KIFAP3, kinesin associated protein 3; NIPA1, nonimprinted in Prader-Willi/Angelman syndrome 1; PGRN, progranulin.

has an aberrant subcellular distribution in NSC34 cells, and cells expressing the mutant protein are less resistant to apoptosis induced by ER stress (9).

In a study conducted on 1948 sALS and fALS cases and 2002 controls from Belgium and The Netherlands, intermediate ($\geq$32, range 32–39) CAG repeat expansions in ataxin 2 (ATXN2), the causative gene of spinocerebellar ataxia type 2 (SCA2), have been found in sALS and in unexplained fALS, thus indicating a possible genetic overlap between ALS and SCA2 (544).

Finally, two independent studies found an association of ALS-FTD with an expansion of a noncoding GGGGCC hexanucleotide repeat in the gene *C9ORF72* (126, 442).

Strikingly, the frequency of this repeat expansion makes this the most frequent genetic cause of fALS and FTD, ranging from 12% of familial FTD and 22.5% of fALS in North America, 46% of fALS, 21.1% of sALS, and 29.3% of FTD in the Finnish population, to about 30% in an outbred European population (410).

Three C9ORF72 transcripts are produced through alternative splicing in a variety of tissues, including the brain, one of which is decreased in the GGGGCC repeat carriers (126). The protein encoded by this ORF seems to be predominantly localized within the nucleus in human control fibroblast cell lines and in the mouse motorneuronal NSC-34 cell line (442), but its function is not known.

In about 25% of cells in the frontal cortex and the spinal cord from patients carrying the expansion, the transcribed GGGGCC repeat forms nuclear RNA foci (126), that is, the intracellular accumulation of RNA fragments composed of the expanded nucleotide repeats which seem to represent an important disease mechanism for the class of noncoding repeat expansion disorders (525).

Interestingly, the GGGGCC sequence represents a potential binding site of several RNA-binding proteins, including the hnRNP A2/B1 that has been shown to interact directly with TDP-43 (65, 485), and RNA generated from pathogenic repeat expansions such as GGGGCC are thought to disrupt transcription by sequestering normal RNA and the proteins involved in transcription regulation (586).

Other possible ALS genes have been identified by genome-wide association studies and are listed in Table 3.

It is clear that at least some of the genes just mentioned need to be conclusively confirmed as true ALS genes. Nonetheless, the picture of the mechanisms involved in the disease that emerges from these studies is strikingly different from the one we had only a few years ago when the conclusive association of mutant SOD1 to fALS pointed to oxidative stress as a major player in the disease.

### *B. Gene expression in ALS*

Whatever the genotype of the patient and despite a certain heterogeneity in symptoms presentation and progression, fALS and sALS share largely overlapping clinical features, and progressive MN degeneration and muscle waste are common to all ALS patients. Thus, it is plausible that a set of alterations in gene expression that are common to all forms of ALS may be found, which may aid in understanding molecular mechanisms and in providing therapeutic targets and biomarkers. Several approaches have been employed in this field, from studies aimed at the identification of epigenetic modifications and post-transcriptional regulation of mRNA translation to the determination of an ALS-specific transcriptome and proteome.

#### 1. Epigenetic regulation of mRNA transcription in ALS.

Epigenetic alterations of transcription due to an unbalance between the activities pertaining to histone acetyl transferases (HATs) and those of histone deacetylases (HDACs) have been described in a variety of neurodegenerative conditions. In the particular case of ALS, it is currently believed that the equilibrium between the action of the HATs and that of HDACs is shifted toward the deacetylation activity (464).

SIRT1 (an HDAC of the Sirtuin family) is up-regulated and seems to play a neuroprotective role in the G93A-SOD1 mouse model of ALS (292). This was also confirmed in an ALS cell model using resveratrol and nicotinamide, an activator and an inhibitor of SIRT1, respectively (565).

On the other hand, HDAC inhibitors have been tested as potential neuroprotective drugs for the treatment of neurodegenerative diseases, including ALS. Sodium phenylbutyrate, an unspecific inhibitor of HDACs, is able to promote cell

survival in G93A-SOD1 mice (421) and has been employed in a Phase 2 study with encouraging results (114). Valproic acid (VPA), an HDAC inhibitor with antioxidative and anti-apoptotic properties, also has beneficial effects on disease duration in an ALS mouse model (502), and a combined lithium and VPA treatment is effective in delaying the onset of disease symptoms, prolonging the lifespan, and decreasing the neurological deficit scores in the same mice (160). Treatment of G93A-SOD1 mice at the early symptomatic stage with another HDAC inhibitor, trichostatin A, induces a reduction of MN death and axonal degeneration, a decrease of gliosis, an up-regulation of the glutamate transporter, a reduction of muscle atrophy and NMJ denervation, an enhanced motor function, and an increased mean of survival (601). Unfortunately, these promising results found no confirmation in a pharmacological trial performed for VPA in 163 ALS patients (422).

The major limitation of the treatment with the HDAC inhibitors currently available most probably lies in the broad spectrum of the action of these drugs, which may cause a variety of toxic side effects. Thus, understanding which HDACs are really involved in ALS and testing specific inhibitors or activators should yield precious information. This has been attempted in a study analyzing HDACs mRNA expression levels in human postmortem tissues, which revealed a reduction in HDAC11 mRNA and an increase in HDAC 2 levels in ALS brain and spinal cord compared with controls (262); no data on the protein levels or enzyme activity were provided.

It is interesting that complexes formed by TDP-43 and FUS/TLS control the expression level of HDAC6 (294), suggesting that the alteration of the activity of this protein could mediate the toxic action of the FUS or TDP43 mutation in inducing ALS.

Thus, epigenetic alterations of gene expression may represent a feature shared by different forms of fALS.

2. Post-trascriptional regulation of mRNA expression in ALS. A different method of modulating gene expression is the regulation by microRNA (miRNA). A crucial role for miRNA-dependent post-transcriptional gene regulation in the development and function of neurons is largely documented, along with the observation that the alteration in the function of miRNA contributes to susceptibility to neuronal disease (148).

In 2009, Williams *et al.* reported that miRNA-206 is dramatically induced in mutant SOD1 mice and that this up-regulation coincides with the onset of the disease. In this article, they also demonstrated that miRNA-206 is able to deeply influence the process of NMJ regeneration after injury and that the loss of miR-206 does not affect disease onset but rather accelerates disease progression with an accelerated atrophy of skeletal muscle, paralysis, and death. Thus, miRNA-206 may be considered a modifier of ALS pathogenesis (583).

A further indication of a possible role of miRNAs in ALS comes from the observation that TDP-43 is able to control the synthesis of specific miRNA and, thus, modify several transcripts whose expression levels are affected by these TDP-43-miRNA interactions (66).

Thus, it will be interesting to learn whether miRNA are involved in other genetic or spoeadic forms of ALS.

3. ALS transcriptome. That the overall pattern of gene expression is modified in ALS is indicated by several studies addressing transcriptomics and proteomics of ALS models and tissues. The availability of powerful genomics technologies as microarrays analysis has provided the opportunity to reveal complex regulatory and interactive pathways controlling neuronal phenotypes and changes in the expression levels of proteins. After a few early studies conducted on mRNA from the whole spinal cord from patients (349), from G93A-SOD1 transgenic mouse (408, 602), and from NSC34 cells (296), more refined whole-genome expression profiling studies in the motor cortex of seven sALS patients suggested that most of the deregulated genes are involved in defense responses, cytoskeletal dynamics, and mitochondrial dysfunction (323). In line with these findings, in an elegant study using laser-capture microdissection and microarray analysis that allowed an analysis of changes specifically in MNs and still interacting with neighboring cells, Ferraiuolo *et al.* described the pattern of gene expression in MNs from G93A-SOD1 mice (163). In these cells, the analysis of up-regulated genes suggests the activation of a strong cellular adaptive response in the asymptomatic mice, which gradually changes to marked transcriptional repression and up-regulation of complement system components and of key cyclins late in the disease course. More recently, Offen and coworkers have analyzed the mRNA gene profiling of the spinal cord of patients with sALS and compared it with the profile of G93A-SOD1 transgenic mice. The major changes were found in 60 genes, including cathepsin B and cathepsin D, apolipoprotein E, epidermal growth factor receptor, ferritin, and lysosomal trafficking regulator (404).

With the aim of analyzing the modification of mRNAs levels during the human disease, Saris *et al.* investigated changes in gene expression profiles in the whole blood of ALS patients. The study showed an alteration of 2300 transcripts, and the Ingenuity Pathway Analysis indicated an enrichment of the functional categories related to genetic disorders, neurodegeneration of the nervous system, and inflammatory disease (458).

To identify the proteins involved in gliosis, which concurs to neurodegeneration, an RNA microarray analysis has been also performed on astrocytes from the spinal cord of G93A-SOD1 mice. Decorin, a small multifunctional extracellular proteoglycan, has been identified as the most up-regulated gene, while the down-regulated genes included the insulin-like growth factor-1 (IGF-1) receptor and the RNA-binding protein ROD1 (554).

The first study that allowed the identification of a major set of gene regulations in muscles from G86R-SOD1 mice (202) was followed by a similar study which aimed at the identification of gene expression changes in the skeletal muscle of ALS patients that could reliably define the degree of disease severity. From the analysis of functionally related transcripts that appear differentially regulated exclusively in early and advanced states of deltoid muscle impairment, nine transcripts allow prediction of the advanced disease status with 100% sensitivity and specificity and, thus, can be of practical use when monitoring ALS status and the effects of disease-modifying drugs (427).

On the whole, these studies point to a (relatively high) number of candidate pathways that may be affected by the

disease. It is clear that further studies are needed to discriminate between relevant pathways and "background noise" in patients.

4. ALS proteome and biomarkers. Trascriptomic analysis has been paralleled by a proteomic analysis approach which aims at identifying the alterations of protein expression levels or the production of post-transduction modifications of specific proteins that may play a crucial role in the pathogenesis of ALS. Proteomic analysis has been performed in cellular and animal ALS models (43, 75, 136, 343, 355) and also in patients (59, 215) in an attempt to identify pathogenic alterations or useful disease markers (56). One of the most recent and complete analysis was performed in spinal cord proteins from G127X-SOD1 mice (42) and led to the identification of 54 differentially regulated proteins, which are mostly neuron specific and correlated with oxidative stress, modifications of filament dynamics and the protein degradation system, and related to alterations in mitochondria, in agreement with previous studies. A specific proteomic study of mitochondrial fraction from G93A-SOD1 rats, partly replicated on human purified mitochondria from patients, has indicated that the alterations mostly affect Complex I activity and the efficiency of mitochondrial import pathways (332).

Another aspect of the proteomic approach in ALS consists of the analysis of the protein content in cellular inclusions that are detectable in most ALS forms. Two different articles have provided the proteomic characterization of protein inclusions in SOD1-fALS; in one study in spinal cords from two different mouse models (43), mutant SOD1 accounted for half of the proteins contained in insoluble inclusions, with 10 other proteins identified as 2 cytoplasmic chaperones, 4 cytoskeletal proteins, and 4 proteins that usually reside in the ER. In the other study (32), an analysis of the insoluble fraction was performed in the spinal cord from G93A-SOD1 mice at different disease stages and from patients. This study identified several proteins enriched in the detergent-insoluble fraction already at a preclinical stage, including intermediate filaments, chaperones, and mitochondrial proteins such as aconitase, HSC70, and cyclophilin A, and also supported a role for nitrative stress and protein nitration as contributors to the process of aggregate formation.

Many of the proteomic studies in the field of ALS have as a target the discovery and validation of biomarkers of the disease that are easily detectable from tissues or biological fluid from patients [see for review (454, 538)]. These studies have been performed from different tissues, including CSF, blood serum or plasma, urine, bioptic muscle tissue, or postmortem spinal cord tissue samples. Due to its close anatomical contact with the brain and spinal cord interstitial fluid, the CSF is considered the site where biochemical alterations related to a chronic neurodegenerative disease are likely to be reflected and accumulate and, thus, CSF is widely used (56, 194, 507, 538, 604), but no univocal marker has been reliably identified so far.

A different proteomic approach that is used for the identification of biomarkers consists of the search for biomarker candidates in a model system such as transgenic mice, followed by a validation in ALS patients (393). These studies will possibly be extended in the next future.

## III. ALS Is a Multifactorial Disease

Considering the wide spectrum of genetic abnormalities responsible for or associated to ALS, it turns out that the common clinical features found in ALS patients may descend from the concurrence of yet unidentified environmental factors which intervene to make different pathogenic mechanisms converge in a final common pathway.

A detailed description of several proposed mechanisms has been the subject of a large number of investigations, which are often based on SOD1-linked models and reported in several extended reviews in the past (107, 251, 449). Here, we will provide an update, with a focus on new angles and perspectives that the discovery of new disease genes has opened in this field.

### *A. Protein aggregation and impairment of axonal transport*

Protein aggregates are frequently found in spinal MNs of all types of ALS patients; they consist of ubiquitinated skein-like inclusions, Bunina bodies, and hyaline inclusions. These inclusions contain many different proteins, some of which may have an intrinsic tendency to aggregate after mutation (such as SOD1, TDP-43, FUS/TLS, and OPTN), while others may be simply entrapped in aggregates (107).

In 1993, due to the early discovery of the linkage between SOD1 mutations and fALS, experimental models based on these mutations were the first to be developed (38) and are still widely used, despite some criticism based on the consideration that to date a number of treatments increasing the survival of transgenic mice expressing the mutant SOD1 has given disappointing results in clinical trials. The failure to translate results obtained in those mice into an effective therapy for patients, however, may depend on a number of factors (73, 342, 467) and rather than simply discourage the use of models based on mutant SOD1 serves as a lesson that better designed studies may greatly help (362) and that parallel studies in models based on other mutant proteins are needed.

As just mentioned, the idea of a loss of superoxide scavenging function was discarded soon after the first reports of mutations in a subset of ALS cases, and there is now a general consensus that the toxic function gained by the mutant enzymes is related by their propensity to aggregate and to mislocalize, analogously, to other ALS mutant proteins.

This hypothesis well fits with a prominent characteristic that is observed in ALS patients and is almost always recapitulated in models of the disease: the presence of intracellular inclusions enriched in the SOD1 protein in the cytosol (but also in mitochondria) of model cells and, of course, in spinal cord MNs in animals (81, 107). During the last years, copious computational and experimental evidence led to the hypothesis that mutations in SOD1 decrease dimer stability, and/or increase dimer dissociation (287, 288), eventually leading to misfolding and aggregation, which is, to date, the most conceivable unifying way of explaining how about 150 different mutations in the SOD1 polypeptide are responsible for the same disease (81). Moreover, it has been indicated that the increased aggregation propensity of SOD1 mutants is related to the decreased survival of ALS patients, further suggesting that aggregation could be a major contributor to SOD1 toxicity in ALS patients (431, 572).

Some recent findings have added weight to the major role of protein aggregation in the disease. Misfolded SOD1, identified by a specific antibody, was found in tissues from both sporadic and familial ALS cases, but without SOD1 mutations, and the same antibody recognizes oxidized wild-type SOD1 *in vitro* (53), thus reinforcing the idea that a common pathway of protein misfolding and aggregation underlies sALS and fALS, as had been put forward by previous evidence (155, 215). Moreover, the co-expression of wild-type SOD1 with mutant SOD1 is able to exacerbate the disease phenotypes in most (130, 131, 258, 566), but not all (22), transgenic mice, thus indicating that the presence of wild-type SOD1 can affect the clinical and patologycal outcomes of the disease.

Indeed, mutant SOD1 is destabilized but relatively prone to SOD1-SOD1 aggregation (493), and the effects of mutant SOD1 in ALS are at least partially mediated through heterodimer formation with WT SOD1 (585).

Despite all these considerations, different lines of evidence suggest that SOD1 aggregation does not play a primary role in the disease. Although previous articles have suggested that the accumulation of insoluble, aggregated mutant SOD1 is an early indicator of the disease (31, 258, 461, 564), it has been recently shown that the accumulation of large aggregates of mutant SOD1 seems to occur primarily in late stages of the disease of G93A-, G37R-, and H46R/H48Q-SOD1 transgenic mice, concurrently with the appearance of rapidly progressing symptoms (278). The overexpression of copper chaperone for SOD1 (CCS) in G93A- and G37R- (but not in G85R- and L126Z-) SOD1 mice accelerates neurological disease without enhancing the deposition of SOD1 aggregates which are undetectable at the time mice die, thus indicating that aggregates are not the driving force of the disease (430, 486) and neuronal overexpression of chromogranin A accelerates disease onset in the G37R mouse model of ALS, without enhancing SOD1 aggregation (154).

All these results shift the attention to other forms of misfolded mutant SOD1 that could be actually involved in the disease. Among them, oligomers or misfolded monomers, which might be on the verge of protein aggregation, have been proposed to be the toxic species (468), and different forms of misfolded mutant SOD1 have been detected in tissues from models and patients using conformation-specific antibodies that have been developed during the last years (436, 550, 551, 604). Interestingly, an antibody against misfolded forms of mutant SOD1 delays mortality in the G93A-SOD1 mouse model of the disease, thus indicating that misfolded mutant SOD1 is responsible for the disease phenotypes in mice (211).

Using such a type of antibodies, which specifically recognizes residues 42–48 of SOD1 in which the $\beta$-barrel is unfolded, Kerman *et al.* have investigated the *in vivo* conformation of SOD1 in tissues from sporadic and SOD1-linked ALS patients, and concluded that ALS is a non-amyloid disease, in most instances, and extensive misfolding of SOD1 occurs uniquely in the familial form (285). However, these observations fit neither previous data from *in vitro* studies showing that wild-type or mutant SOD1 form amylod-like structures and helical filamentous arrays that have some similarity to the amyloid structures which characterize other misfolding diseases (80, 149, 321), nor previous observations from transgenic mice showing the presence of amyloid-like material (563), and, thus, the features of SOD1 aggregates that form *in vivo* remain poorly understood.

The fact that protein aggregation is a common theme in ALS and that the high specificity of cellular degeneration in ALS depends on the sensitivity of MNs to protein aggregation is also suggested by the observation that, similarly to SOD1, also TDP-43, FUS/TLS (and OPTN and ubiquilin 2 as well), aggregate in tissues from ALS patients and models (345).

Indeed, anatomo-pathological observations of ALS-affected tissues showed that these two proteins abnormally aggregate as cytoplasmic inclusions which are positive for ubiquitin, a characteristic feature of the inclusions of degenerating MNs in ALS, but negative for SOD1 (Table 2).

Both TDP-43 and FUS/TLS are proteins that can aggregate, and ALS-related mutations seem to enhance the rate of aggregation (216, 264, 399, 504), as observed in transfected NSC34 cells (Fig. 1, our unpublished results). However, using a yeast model that tests the effects of ALS-linked mutations on FUS/TLS aggregation and toxicity, Sun *et al.* found that FUS/TLS mutations do not necessarily promote the aggregation of the protein, suggesting that the aggregation of this protein can be secondary to other pathology-relevant effects (*i.e.*, nuclear localization impairment).

Interestingly, a novel prion-like domain has been identified in FUS/TLS (a.a. 1–232 and 391–405) and TDP-43 (a.a. 277–414). Similar to other prion domains, this domain is enriched

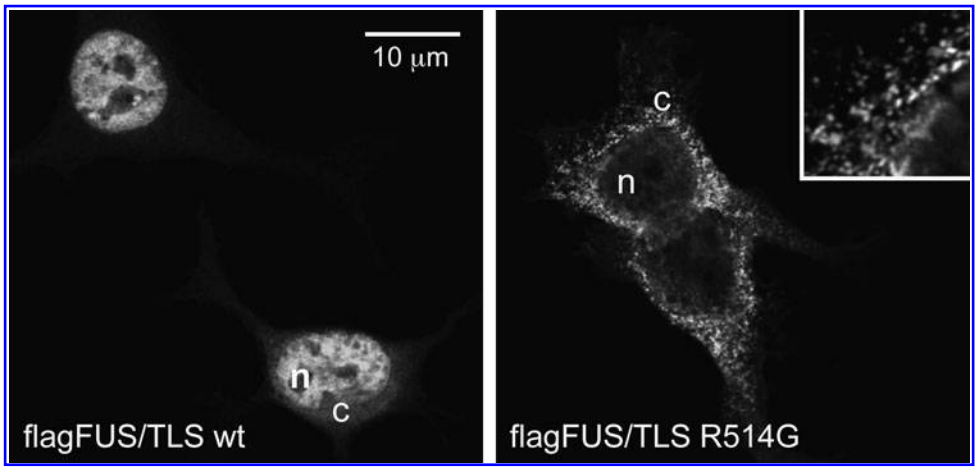


**FIG. 1. Cytoplasmic mislocalization and aggregation of amyotrophic lateral sclerosis (ALS)-linked mutant fused in sarcoma/translocated in liposarcoma protein (FUS/TLS).** Mouse motoneuronal NSC34 cells were transiently transfected with Flag-tagged wild type FUS/TLS or the R521G mutant. Overexpressed proteins were labeled with a fluorescence-conjugated secondary antibody. *Inset* is a higher magnification of a selected region. c, cytosol; n, nucleus.

in uncharged polar amino acids and glycine, and it seems to be required for the aggregation of both the proteins. In both ALS and FTD patients, as well as in *in vitro* experiments, TDP-43 is found as accumulated C-terminal fragments of ~35 to ~25 kD. These fragments are hyperphosphorylated and they have been proposed to be generated from proteolytic cleavage by caspases, due to the presence of caspase consensus cleavage sites (17, 609, 610). Both the C-terminal and the N-terminal fragments of TDP-43 can form intracellular inclusions, and the aggregation of the TDP-43 fragments can drive co-aggregates with the full-length TDP-43, thereby reducing nuclear TDP-43 (595). Whether seeds of aggregation can propagate further aggregation of TDP-43 or FUS/TLS is to be established. Interestingly, two recent studies have provided evidence of a prion-like propagation of the aggregation of mutant SOD1. Aggregates prepared from recombinant SOD1 are able to enter the cytosol through a macropinocytotic process, and to nucleate the aggregation of the pre-existing cytosolic soluble form of mutant SOD1 (387). Moreover, spinal cord homogenates obtained from the G93A-SOD1 transgenic mouse contain amyloid seeds that accelerate the formation of new fibrils in both wild-type and mutant SOD1 protein *in vitro* (90). Since aggregates are released by cells and uptaken by nearby cells, transmission of the misfolding pathology is established and self-propagated, similarly to what happens in prion disorders (432), and this modality might also explain the long phase of latency and the rapid progression that frequently characterize the disease.

The formation of such intracellular aggregates may depend on the accumulation of misfolded proteins generated either as a direct consequence of mutation, or as a consequence of oxidative stress. In both instances, impairment of the ubiquitin-proteasome response (UPR) seems to play a major role. Not only ubiquitin-immunoreactive inclusions are the most frequently reported inclusions in all forms of ALS, but also aggregates may be reactive to dorfin, a RING-finger type E3 ubiquitin ligase (185, 253, 376), and p62, a protein that has a part in the formation of the sequestosome and in autophagy (27, 469). The proteosomal activity is inhibited in mutant SOD1 models (541), and SOD1 is mono- and oligo-ubiquitinated in the mutant SOD1 mouse model. However, ubiquitination occurs only after SOD1 aggregation and, thus, may represent a secondary event that possibly affects proteasomal function (31). More evidence on the involvement of defective protein removal in ALS comes from the observation of altered function of the UPR in MNs of the SOD1 mouse model of ALS (89), which is accompanied by the activation through the small heat-shock protein B8 (HspB8) of the autophagic pathway, to increase mutant SOD1 clearance (462).

Aggregated TDP-43 is also polyubiquinated and degraded by both proteasome and autophagy (154, 575). The autophagosomal delivery and/or proteasome targeting of mutant TDP-43 aggregates is mediated by the presenilin-binding protein ubiquilin 1 in yeast (295), and the co-expression of ubiquilin 1 in *Drosophila* reduces steady-state TDP-43 expression but enhances the severity of TDP-43 phenotypes (224).

The failure of protein quality control in ALS is also indicated by a number of articles reporting ER stress in mutant SOD1 models (277, 289, 600) and in spinal cord tissues of human sALS patients (250), and recent evidence suggests that perturbation of the ER could occur in ALS cases associated with TDP-43, FUS, and VCP (562), along with an activation of the unfolded protein response (569), a process in which the candidate ALS gene VAPB is suspected to play a role.

A detailed description of the alteration of the secretory pathway (including a failure in the protein folding machinery in the ER, activation of UPR, modifications of the Golgi and impaired vesicular trafficking, inhibition of protein quality control mechanisms, and persistent activation of autophagy) that occurs in ALS has been the subject of recent reviews (394, 562).

Protein aggregates may result toxic for MNs, either because they entrap proteins that are critical for their viability or as they cause a mechanical hindrance and impairment of axonal transport. Indeed, defects in axonal transport have been well documented in the SOD1 mouse model [for a review (348)] and clearly precede the onset of symptoms in these mice (46). Furthermore, among previously proposed candidate genes, some code for proteins belonging to the neurofilaments (NFLs) family and to the dynein-dynactin machinery that is involved in neuronal transport (107).

### B. Excitotoxicity

Glutamate excitotoxicity resulting from repetitive cell firing or the influx of excessive levels of calcium through glutamate receptors was one of the first proposed mechanisms of MN degeneration in ALS, on the basis that increased levels of glutamate were observed in the CSF of ALS patients (450). A number of later studies support the hypothesis that selective impairment of the astroglial glutamate transporter (excitatory amino acid transporter 2 [EAAT2]) contributes to MN degeneration in ALS (175, 449).

EAAT2 is sensitive to oxidative stress (533) and is cleaved by caspase-3 at its cytosolic carboxy-terminus domain (54). This cleavage generates a sumoylated proteolytic fragment (195) that accumulates in the nucleus of spinal cord astrocytes of the G93A-SOD1 mice and may result toxic (174). However, the accumulation of this fragment occurs at symptomatic stages of the disease, in line with a previous report demonstrating that the same mice experience a significant decrease in the level of that transporter only at the advanced stage of the disease (39). This casts some doubt that the loss of EAAT2 is a primary cause of ALS, and rather suggests that EAAT2 may contribute only to the progression of the disease.

With the exception of the rare mutations in nAChR subunits (see above), recent evidence on the genetics of fALS does not contribute much toward corroborating the concept of glutamate dys-metabolism as a major player in the pathogenesis of this disease. Conversely, new emphasis is placed on the mishandling of intracellular calcium after mitochondria and ER alterations. This is described in greater detail in the next few paragraphs.

### C. Organelle dysfunction

1. Mitochondria. It has long been known that dysfunctions of mitochondria are involved in the pathogenesis of ALS. Indeed, the pathological phenotypes of ALS well correlate with alterations in most of the processes of mitochondrial function, that is, morphology and bioenergetics, transportation and clearance, apoptosis and calcium buffering (106). These last years have witnessed a strong improvement in our knowledge of the possible mechanisms whereby

mitochondrial functions might be affected, as will be discussed in the next few paragraphs, but the central question of whether, and to which extent, mitochondrial dysfunction is a primary target of the disease-causing mechanism has not been fully addressed.

*a. SOD1 localization and import into mitochondria.* A mechanistic insight into the ALS-causing property of mutant SOD1 is suggested by its association to mitochondria. SOD1 is predominantly cytosolic, yet a small but considerable part of the enzyme resides in mitochondria. This stems from pioneering articles by Fridovich (407, 579), and has found decisive confirmations in articles by many others (239, 257, 356). In particular, mitochondrial SOD1 is enriched in the intermembrane space, with a small portion also detected on the cytoplasmic face of the outer membrane (338) and in the matrix (556).

Although the biological function of this fraction of the enzyme has not been fully addressed, different observations suggest a protective role of SOD1 in this compartment. Mitochondrial SOD1 protects yeast mitochondrial proteins from oxidative damage (298, 401), and decreasing SOD1 with the help of small, interfering RNA has been shown to lead to severe damage to the mitochondria of human neuroblastoma cultured cells (16) and to a robust increase in the oxidation of mitochondrial proteins. Most importantly, when wild-type SOD1 are targeted, through an artificial mitochondrial targeting signal, to the outer side of the mitochondrial inner membrane of mice with an Sod1(−/−) background, the biochemical and morphological defects that characterize the Sod1(−/−) animals are prevented, and the motor phenotypes of these mice are rescued (172). These results suggest that SOD1 in the mitochondrial intermembrane space prevents mitochondrial oxidative damage and is fundamental for motor axon maintenance.

SOD1 lacks a canonical mitochondrial targeting sequence (MTS); thus, it apparently escapes the main route of protein import which characterizes the vast majority of mitochondrial protein, that is, crossing the outer membrane *via* the translocase of the outer membrane complex, and sorting to different mitochondria subcompartments, due to the presence of either amino-terminal amphipathic presequences, which are cleaved from the protein during the import process, or internal targeting sequences, which remain a part of the fully matured protein (465). For this reason, the mechanism of SOD1 import in mitochondria has remained elusive for a long time, although CCS was earlier identified as being central to this mechanism (168).

However, in the last years, decisive articles characterized a disulfide relay system that is operative in the intramembrane space (IMS) of mitochondria and which drives the import of a set of mitochondrial proteins lacking MTSs, through their oxidative folding (364). In particular, Mia40, an oxidoreductase, forms a mixed disulfide intermediate with the substrate protein and then transfers the disulfide bond to the substrate, and Erv1, a sulphydryl oxidase, reduces Mia40 and shuttles the electrons to the electron transport chain. At the end of the process, the oxidized, folded protein is entrapped in the IMS of the mitochondria (479). CCS has been recently demonstrated to be a relevant target of the Mia40/Erv1 pathway of protein import (212, 283, 299, 440). The amount of imported CCS is affected by the respiratory chain, is sensitive to oxygen concentrations (283, 440), and, in turn, orchestrates the transport of SOD1 inside the IMS by promoting the folding of the protein, which is oxidative in nature as well. As expected, Cys57 and Cys146 of human SOD1, which form an intramolecular disulfide bridge that is essential for the folding of the protein, play a role in the accumulation of SOD1 in the mitochondria, while Cys6 and Cys111 may play a role in the disulfide bridging to the proteins that are involved in the import mechanism (164, 283).

All mutant SOD1s analyzed so far, in both cellular and animal models of the disease, were found to be variably associated to mitochondria, indicating that mutations do not affect *per se* the overall import of the protein (44, 131, 164, 239, 254, 257, 338, 417, 551, 556). Rather, misfolding of the protein, which is variably induced by mutations, might cause the aggregation inside the mitochondria of these mutants, which would eventually escape the regulation of the mitochondrial localization of SOD1 by CCS, and so would not properly respond to the physiological needs of the mitochondria (283). Indeed, different lines of evidence suggest that mutant SOD1s associate with mitochondria in a non-native, oligomeric, and aggregated state (131, 164, 165, 184).

Although the pathological relevance of mutant SOD1 localization in mitochondria has been questioned (44), evidence suggests that this localization might play a direct role in cell dysfunctions in ALS. Mutant SOD1s that are targeted to the mitochondria of cultured cells by ectopic mitochondrial targeting signals induce mitochondrial damage and cell toxicity, thus providing the proof of principle that mitochondrial localization of mutant SOD1 is sufficient to cause ALS-like phenotypes (108, 347, 515). Moreover, the overexpression of the mitochondrial isoform of glutaredoxin, Grx2, decreases disulfide-linked oligomers by mutant SOD1, increases its solubility, preserves mitochondrial function, and strongly protects cultured neuronal cells from apoptosis (165), thus suggesting that the toxicity of mutant SOD1 mostly arises from mitochondrial localization/aggregation of the protein. Most importantly, mitochondrial targets that are directly affected by mutant SOD1s are being characterized. Among them are Bcl2, an active partner in mutant SOD1-induced mitochondrial toxicity (417, 419); the mitochondrial form of lysyl-tRNA synthetase (KARS), an enzyme required for protein translation that was shown to interact with mutant SOD1 in the mitochondria of mamalian cells and transgenic mice and to misfold and aggregate in the presence of mutant SOD1, a condition that might impair the activity of the protein and/or its import inside the mitochondria (282); and the VDAC1, an integral membrane protein of the outer mitochondrial membrane that has been recently shown to specifically transfer $Ca^{2+}$ apoptotic signals to the mitochondria (123), is bound by misfolded mutant SOD1. The binding causes a decrease in the activity of this protein, with a plausible detrimental effect on spinal MNs, in which VDAC1 appears to exert a protective role (254). Indeed, all the examples just outlined put forward the hypothesis that a prominent toxic activity of mutant SOD1 derives from its ability to interact with nuclear-encoded mitochondrial proteins, thereby hindering their normal shuttling between the cytosol and the mitochondria. In this context, it should be noted that mutant SOD1 was recently shown to alter the protein import in mitochondria of the rat spinal cord (332), thus affecting the mitochondrial protein content.

Thus, one would expect that targeting mutant SOD1 to the mitochondria of mice would recapitulate, or even exacerbate, the ALS phenotype. Indeed, altered mitochondrial bioenergetics, as well as reduced spinal MNs, body weight loss, muscle weakness, brain atrophy, and motor impairment characterize mice in which mitochondria-targeted G93A mutSOD1 is overexpressed (247). However, muscle denervation, a central feature of ALS pathology, is not induced in these mice, thus indicating that mitochondrial accumulation does not account for all the toxic properties of mutant SOD1 described so far. Other signals, therefore, originating from pools of mutant SOD1 localized in other cellular compartments are probably needed.

*b. Mitochondria and oxidative stress.* Increased levels of "classical" markers of oxidative stress, such as reactive oxygen species (ROS) and products of protein and lipid oxidation, have been repeatedly and consistently observed in tissues from patients and in models based on the expression of mutant SOD1 *in vitro* and *in vivo* [for a review (29, 107)].

More recent studies have extended the range and somehow enlarged the concept of markers of oxidative stress in ALS to include alterations linked to cysteine oxidation and glutathione dysmetabolism (32, 164, 165, 553), membrane fluidity (366), and induction of oxidative stress-responsive pathways such as activation of the Bcl-xL gene *via* the Ets-2 transcription factor (324), up-regulation and oxidation of DJ-1 (329), disruption of redox-sensitive Rac regulation of NADPH oxidase in astrocytes (225), and up-regulation of Rad, an inhibitor of voltage-gated calcium channels (222).

The origin of oxidative stress in ALS is still not clear. In the case of fALS linked to mutations in SOD1, the initial idea of a loss of function was demonstrated as being untrue quite early, and the idea that defective SOD handling may occur in non-SOD1 ALS seems unlikely in the light of the known function of other ALS genes. In analogy to other neurodegenerative conditions, oxidative stress in ALS may be caused by a misbalanced metabolism of iron (72). This may be due to the dysregulation of proteins involved in intra- and extra-cellular iron transport; to iron accumulation in MNs caused by defective anterograde axonal transport; and to increased mitochondrial iron load in neurons and glia (263). Indeed, increased iron level and deposits have been reported in some regions of the brain from ALS patients (280, 317, 403, 598), although other magnetic resonance imaging (MRI) studies do not confirm this evidence (228). Iron accumulation has also been reported in neurons and glia in mutant SOD1 transgenic mice (263).

ALS patients have significantly higher levels of H- and L-ferritin in the brain (280), increased levels of L-ferritin in serum and in plasma (203, 374, 433), and lower concentrations of transferrin in the plasma (374), but they do not differ from control subjects with regard to the levels of serum iron, transferring, and total iron-binding capacity (203). Ferritin also is up-regulated in SOD1-G93A mice just before end-stage disease (408). Intriguingly, we observed that levels of the transferrin receptor, the iron regulatory protein 1, and ferritin expression were modulated in response to altered intracellular levels of SOD activity, carried out either by wild-type SOD1 or by an SOD-active ALS mutant enzyme in glial and neuronal cells (120).

Treatment with iron chelators seems to be beneficial in the mutant SOD1 mice (306, 573), and this may be due not only exclusively to the suppression of the Fenton chemistry through the neutralization of excessive reactive $Fe2+$ but also to the inhibition of the iron-dependent hypoxia-inducible factor (HIF) prolyl 4-hydroxylases that regulate HIF stability, leading to the up-regulation of protective genes (477).

Mitochondria are the major sites of formation of ROS, in both physiological and pathological conditions, and mitochondria themselves are particularly susceptible to oxidative stress (388). Thus, the ROS generated from damaged mitochondria can pushover mitochondrial damage, in a vicious cycle that has been repeatedly proposed for neurodegenerative diseases, as well as for other mitochondria-related pathologies (335). In ALS, many observations from cells overexpressing mutant SOD1s support a role for mitochondrial oxidative stress, such as the loss of mitochondrial membrane potential, decreased ATP levels, and increased production of ROS, as measured by a decrease in the ratio between reduced and oxidized glutathione (GSH/GSSG) (18, 41, 164, 363). When the GSH/GSSG ratio is restored, then the mitochondrial function is rescued (165). On the contrary, a 70%–80% reduction in total glutathione, by deletion of the glutamate-cysteine ligase modifier subunit (GCLM−/−), dramatically accelerates the neurological deficit and mitochondrial pathology in the fALS-linked hSOD1 (G93A) mice model, but not in the hSOD1 (H46R/H48Q) mice model, suggesting different disease mechanisms for different mutations (553).

In this context, it is interesting to note that SOD1 misfolding and aggregation is exacerbated by Cys-111 glutathionylation of SOD1, which destabilizes the native homodimer as observed *in vitro* (441) and for SOD1 isolated from human erythrocytes (582). Proctor *et al.* recently described an intermediate state in the pathway to SOD1 dissociation that is stabilized in glutathionylated SOD1as compared with unmodified SOD1 (429). Since glutathionylation, similar to other post-translational modifications, may be induced by oxidative stress, this SOD1 modification could play a role in sALS.

Recently, alterations in specific redox-regulated signal transduction pathways have been proposed as being responsible for the cellular damage observed in ALS models (74). To investigate this issue, we have recently focused our attention on the p66Shc pathway that exerts a central role in cells in the regulation of mitochondrial ROS metabolism, with a possible task in the control of intracellular redox-based signal transduction pathways, and an established role in the control of mitochondrial apoptosis (193). When p66Shc pathway activation is hampered by functionally inactive mutants of the protein, the mitochondrial phenotypes that are evoked in cultured neuronal cells by the overexpression of mutant SOD1s are inhibited, and mitochondrial damage and apoptotic cell death are prevented (Fig. 2). Most importantly, the deletion of p66Shc in the G93A mouse model of the disease ameliorates mitochondrial function, delays onset, improves motor performance, and prolongs survival (420). Although we know neither the mechanism whereby mutant SOD1 induces p66Shc activation, nor whether this mechanism is specific for this disease, yet our observations emphasize the notion that mitochondria redox signaling plays an important role in this disease.

Altogether, it is clear that the (over-) expression of mutant SOD1 brings about both oxidative stress and mitochondrial dysfunction. However, oxidative stress is found in all forms of

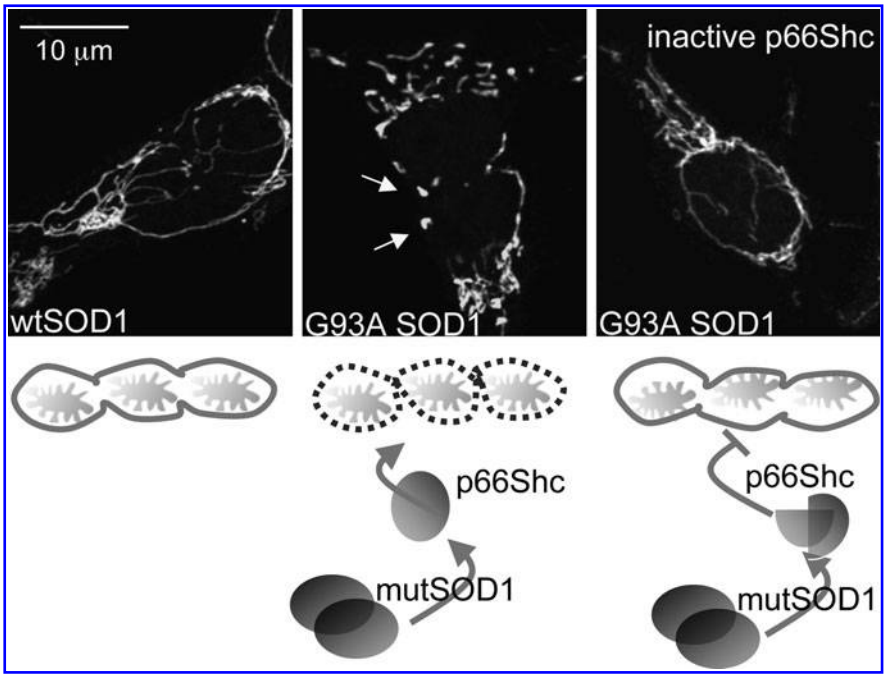


**FIG. 2. Fragmentation of mitochondria by mutant Cu, Zn superoxide dismutase (SOD1).** When mutant G93A-SOD1 is overexpressed in human SH-SY5Y neuroblastoma cells, the filamentous mitochondrial network is lost, and mitochondria appear condensed and fragmented (*white arrows*). When mitochondrial function is preserved by inactivation of the p66Shc mitochondrial redox pathway, normal mitochondrial morphology is restored.

ALS, most of which are not SOD1 dependent. While sporadic forms may be associated to oxidative stress induced by environmental factors (as mentioned in the Introduction section), the link between oxidative stress (invariably found in ALS) and the expression of mutant proteins other than SOD1 is unclear. With the exception of paraoxonases, which have a very well described role in the prevention of lipid peroxidation by oxidative stress (428), not much is known about the possible relations between other ALS-associated mutant proteins and oxidative stress, although interesting data are becoming available for TDP-43. Mutant TDP-43 may be itself an inducer of oxidative stress, as suggested by studies in neuronal cells *in vitro* in which this protein was shown to down-regulate heme oxygenase-1 (141) and in yeast, in which TDP-43 expression increased the markers of oxidative stress and induced cell death (58). On the ground that altered metal ion homeostasis and increased oxidative stress are central features of neurodegeneration, Caragounis and co-workers studied the effect of treatment metals in SH-SY5Y neuronal-like cells expressing endogenous TDP-43. Treatment with zinc induced the depletion of TDP-43 expression and the formation of inclusions that were TDP-43 positive, without evidence of C-terminal fragmentation, phosphorylation, or ubiquitination; these effects were not seen with copper, iron, or hydrogen peroxide (70). C-terminal phosphorylation and fragmentation are produced when oxidative stress is induced by glutathione depletion (248). Oxidative stress also promotes TDP-43 insolubilization and cytoplasmic accumulation, probably through cross-linking *via* cysteine oxidation and disulphide bond formation (96). Overall, these data support TDP-43 as a target of redox signaling.

Furthermore, wild-type TDP-43 is capable of assembling into SGs in response to oxidative stress, and this could be associated with the subsequent formation of TDP-43 ubiquinated protein aggregates, although TDP-43 is not necessary for SG formation (97).

In a cultured cell model, the co-localization of TDP-43 with SGs is controlled by C-Jun N-terminal kinase (JNK), but JNK inhibition does not prevent the formation of Hu protein R (HuR)-positive SGs and does not prevent diffuse TDP-43 accumulation in the cytosol. In contrast, ERK or p38 inhibition prevents the formation of both TDP-43 and HuR-positive SGs (365). The localization of TDP-43 into SGs is also obtained with sorbitol, but wild-type and mutant TDP-43 have distinct stress responses in terms of timing and size of association with SGs (134).

However, SG markers are not entrapped in TDP-43 pathological inclusions in the autoptic spinal cord from three patients affected by sALS (97) and in a recent study, McDonald *et al.* demonstrated that while TDP-43 contributes to both the assembly and maintenance of SGs in response to oxidative stress and differentially regulates key SGs components, two mutant TDP-43 (R361S and D169G) behave very differently as far as this function is concerned (359), thus casting doubt on the relevance of this process in the pathogenesis of ALS.

In addition, FUS/TLS localizes into SGs in response to oxidative stress in inducible HEK-293 cell lines and in zebrafish embryo spinal cords (52), but the relevance of this response in patients is not known. Finally, alsin-deficient mice have an increased sensitivity of neurons to oxidative stress from mitochondrial complex I inhibitors, such as paraquat (68) and SETX defective cells are sensitive to $H_2O_2$ in terms of oxidative DNA damage and enhanced chromosomal instability (505), but again, the relevance of this observation as indicative of a contribution of oxidative stress in ALS needs further confirmation.

*c. Mitochondrial motility, dynamics, and turnover.* A wealth of data have provided indications that ALS-causing conditions, such as mutations in SOD1, affect mitochondria to a different functional level than the one discussed earlier. Indeed, mitochondrial motility (*i.e.*, transport along motoneuronal

axons), dynamics (*i.e.*, fusion and fission), and turnover (*i.e.*, mitophagy) display alterations in animal and cellular models of the disease. Mutant SOD1 perturbs fast axonal transport, reduces axonal mitochondria content in MNs from G93A-SOD1 mice (124), and promotes the accumulation of mitochondria in clusters along the neurites of NSC34 cells (347) and in motor axons of transgenic rats expressing G93A-SOD1 (488). Deficits in retrograde transport have also been identified in presymptomatic ALS mice, indicating that axonal impairment is an early sign of pathology (46) (Fig. 3). Moreover, mutant SOD1 induces morphological alterations in mitochondria that are suggestive of mitochondrial fragmentation (108, 347), and markers of mitochondrial fusion/fission have been recently found to be altered in a cellular model of the disease (165).

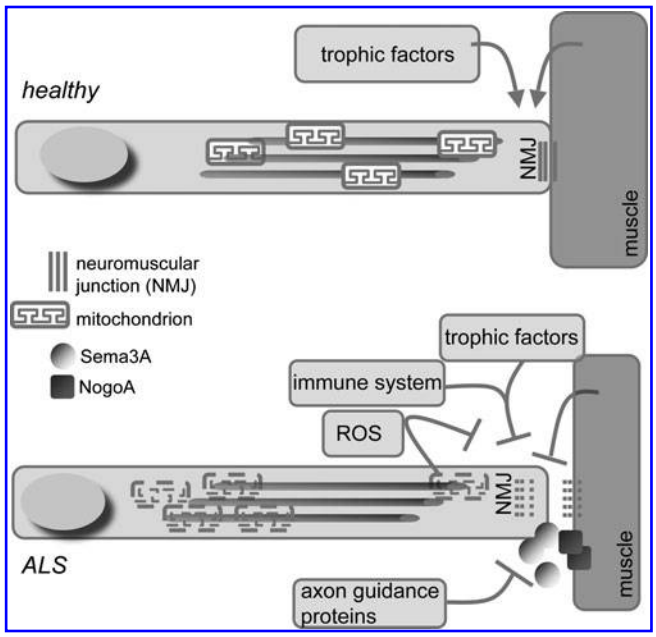


**FIG. 3. The dying-back phenomenon of motor neuron degeneration in ALS**. Retraction of motor axons from innervated muscles is an early event in the presymptomatic phase of ALS and has been indicated as a primary cause of the dying-back degeneration and death of the motor neurons. Different mechanisms might explain this phenomenon. Damage to axonal transport might impair the supply of factors that are necessary for the maintenance of the neuro muscular junction (NMJ) integrity. Moreover, delivery of mitochondria to the cell periphery might be also affected, thereby reducing the amount of available ATP at the distal synapse. This effect would also be reinforced by metabolic dysfunctions that accumulate in the mitochondria as well as increased reactive oxygen species (ROS) production and impairment of mitochondrial calcium buffering. A contribution to the dismantling of NMJs could also be provided by alterations in axon guidance proteins, such as Sema3A and NogoA, which have been described in SOD1 models of the pathology. Further, a role of innate immunity and complement activation in the denervation of the muscle endplate has been proposed. A major role in this phenomenon might also be played by the degeneration of muscle cells, which would be unable to provide trophic and mechanical support to the NMJ. As a consequence, the degeneration of distal synapses occurs, which eventually leads to the death of the motor neurons.

Using a newly generated transgenic mouse line overexpressing a mitochondria-targeted GFP under the mouse Hb9 promoter, which drives gene expression in MN lineage, including a significant proportion of adult MNs, Don Cleveland's group has recently provided a comprehensive view of mitochondrial dynamics in the G37R and G85R mouse models of the disease (550). In particular, they have provided evidence that the misfolded mutant SOD1 is enriched in the mitochondria of motor axons, but not in sensory axons. Moreover, while control mitochondria show a long, tubular morphology and are homogenously distributed along motor axon length, mutant mitochondria are distributed in clusters along the axons and show variable, yet clear disturbed morphologies that are consistent with either increased mitochondrial fission or decreased fusion. Since dys-morphology and mis-distibution of axonal mitochondria is paralleled by the accumulation of misfolded mutant SOD1s along the axons, and considering that defects in both fusion and fission have been shown to decrease the movement of mitochondria (84), the authors have proposed the intriguing hypothesis that misfolded mutant SOD1 impacts the ability of the mitochondria to regulate their shape, which, in turn, influences the correct positioning of the mitochondria at sites where their activity is crucial to MN function. Recently, however, the relevance of mitochondrial transport in ALS has been questioned. Indeed, a two fold increase in axonal mitochondrial motility, which is achieved by deletion of the syntaphilin (snph) gene, is not sufficient to improve disease phenotopes in the G93A mice, suggesting that other features of mitochondrial functions are of primary importance in the pathology (615).

Mitophagy is a prominent method by which the mitochondria are turned over in a cell; it is an autophagic process that is capable of specifically removing damaged mitochondria (33). With regard to autophagy, mitophagy is increasingly attracting the attention of researchers, and, in particular, of neuroscientits, given the role that the dysregulation of autophagy might play in neurodegenerative diseases. Since mitochondrial damage is a sign of ALS pathology, even at early stages, markers of induced or disturbed autophagy/mitophagy are expected to be present in models and patients. Indeed, evidence of induced autophagy has been found in sALS patients and in SOD1 models of the disease (236, 273, 330, 460, 607). In particular, data showing the importance of the autophagic process of misfolded protein clearance have been recently provided. The small HspB8 promotes the autophagic removal of misfolded SOD1 and TDP-43 (110); the deficiency of the x-box binding protein 1 (XBP-1), a transcription factor that regulates the genes involved in protein folding and quality control, unexpectedly protects mice against ALS phenotypes by increasing autophagy and decreasing the accumulation of mutant SOD1 aggregates in the spinal cord, while the inhibition of macroautophagy induces mutant SOD1-mediated cell death. Overall, these observations indicate that macroautophagy reduces the toxicity of mutant SOD1 proteins, although it is not clear why the treatment of G93A-SOD1 mice with the autophagy enhancer rapamycin accelerates MN degeneration, shortens lifespan, and has no obvious effects on the accumulation of SOD1 aggregates (607).

Autophagosomes and autolysosomes containing cytoplasmic organelles, including mitochondria, have been

described in spinal cord MNs in sALS patients (460), but an indepth description of mitophagic phenotypes in models of ALS is still lacking. However, the increased association of PTEN-induced kinase (PINK1) to mitochondria, along with the activation of the autophagy marker LC3, in cultured neuronal cells overexpressing mutant SOD1 (165) is an interesting suggestion that the mitophagic process is activated in ALS models, given that the accumulation of PINK1 to damaged mitochondria is thought to modulate the process of removal of mis-functional mitochondria through autophagy (125) and that PINK1 and LC3 expression is increased in the spinal MNs of G93A-SOD1 transgenic mice (384).

It is possible that mitochondrial damage also occurs in ALS linked to mutant TDP-43 and FUS/TLS. Moderate overexpression of human TDP-43 in mice results in abnormal perinuclear aggregates of mitochondria that are accompanied by enhanced levels of Fis1 and phosphorylated Dlp1, two components of the mitochondrial fission machinery, and by a marked reduction in mitofusin 1, a component of the mitochondrial fusion system (590).

In another mouse model, in which the expression of human TDP-43 is obtained selectively in neurons under control of the Thy1.2 promoter, mitochondria accumulate in the cytoplasm but are absent in motor axon terminals. However, these mice also show alterations in components of the axonal transport system, the kinesin-associated proteins Kif3a and KAP3 (473). Indeed, TDP-43 may indirectly impair mitochondrial transport, stabilizing the mRNA coding for human low-molecular-weight NFL protein (499).

Finally, postmortem examination in two cases of juvenile ALS revealed that abnormal aggregates of FUS proteins and disorganized intracellular organelles, including mitochondria and ER, are found in spinal MNs (244).

*d. Mitochondrial apoptosis.* The involvement of mitochondrial apoptosis in ALS is supported by an extensive literature that has documented a plethora of apoptotic phenotypes in tissues from patients and transgenic mice, as well as in cultured cells overexpressing mutant SOD1s (107). However, the initial interest in apoptotic mechanisms in ALS has declined over the last years, probably because targeting the apoptotic pathway in model mice has not produced the expected results (24, 331, 561), thus casting doubts on the efficacy of an anti-apoptotic strategy for therapeutic intervention, and on the mechanistic role of this process in causing the disease. This conclusion was reinforced by an article by Gould *et al.*, in which G93A-SOD1 overexpressing mice were crossed with mice knocked out for the expression of the pro-apoptotic protein Bax (Bax $-/-$) (205). Bax-deficient G93A-SOD1 MNs were protected from apoptotic cell death, but neuromuscolar denervation was not inhibited, suggesting that apoptosis is not required for the degeneration of MNs, and strengthening the "dying back" pattern of MN dysfunction and the concept that the apoptotic death of neurons occurs only as a later event (171).

However, two recent articles have provided indications that this issue still needs to be developed. Using a similar approach to Gould *et al.*, that is, inactivating the mitochondrial apoptotic pathway by concurrent Bax and Bak deletion in mutant SOD1 mice, Reyes *et al.* have, in fact, shown that an overall organization of NMJs is preserved, and mice are strongly protected from ALS disease, both in terms of symptom onset and survival (443). Moreover, the group of Pasinelli has shown that the interaction of mutant SOD1 with Bcl-2 (417) promotes a conformational change in Bcl-2 which confers a pro-apoptotic property to this protein (419). Once more, these observations, along with recent evidence on TDP-43 models (510), argue for a primary role of mitochondrial apoptosis in ALS and suggest that other relevant pathways regulating mitochondrial apoptosis need to be investigated to fully clarify this topic.

2. Endoplasmic reticulum and Golgi apparatus. The ER calcium signaling in neurons is time-locked to plasmalemmal ion channel activity, such as calcium permeable AMPA receptors, and mitochondria. This interaction has been termed the ER-mitochondria calcium cycle (ERMCC), which couples energy metabolism in mitochondria and protein processing in the ER to the synaptic activity in neurons, and, thus, to total demand in energy and structural maintenance (214). The ERMCC seems to mediate AMPA receptor-mediated glutamate excitotoxicity in motoneurons where a toxic shift of calcium from the ER compartment to the mitochondrial compartment causes chronic calcium depletion of the ER with exhaustion of the protective UPR, and chronic mitochondrial calcium overload with consecutive release of cell death signals through the mitochondrial permeability transition pore. Evidence is accumulating that the ER plays a crucial role in the pathology of ALS (394).

*a. Morphological alterations of the ER and Golgi apparatus.* In mice with the G93A-SOD1 mutation, a dilatation of the rough ER (rER) accompanied with ribosomal detachment was described in pre- and early- symptomatic stages (118). In postmortem ALS spinal cord, fragmentation of the rER, an irregular distension of rER cisternae and a detachment of ribosomes (413), and amorphous or granular material in the ER lumen (459) were found. Fragmentation of the Golgi apparatus was found both in patients post mortem (182) and in mutant SOD1 mice during the symptomatic and presymptomatic stage (118, 386, 496, 497). These changes can be interpreted as a general dysfunction of the ER-secretory pathway and the protein degradation processes in ALS that have been comprehensively reviewed in detail elsewhere (394). This view is supported by the fact that (as mentioned in the Genetics of ALS section) several other ALS proteins (VAPB, FIG4, OPTN, CHMP2B, and, possibly also, Alsin, VAPB, and VCP) and the recently discovered ubiquilin 2 seem to play a role in either organelle trafficking or the control of the degradation of ubiquitinated proteins.

*b. ER stress and its sensors.* ER stress occurs in the presence of mutant proteins that cannot be properly folded. Mutations of SOD1 cause a retention of the cytosolic SOD1 protein in the ER (291, 524, 576). ER stress can also be induced by a disturbance of the ER calcium homeostasis (Fig. 4), likely because protein processing and protein folding are strongly calcium dependent. ER activity is locked to mitochondrial calcium homeostasis and cytosolic calcium transients through the ERMCC (see next). MNs are more prone to saturation of the mitochondrial calcium buffer compared with non-MNs (213), likely due to a lower spacial density of mitochondria in MNs. In addition, mitochondria from G93A-SOD1 mouse spinal cord have a reduced calcium loading capacity (119). In G93A-SOD1 SH-SY5Y

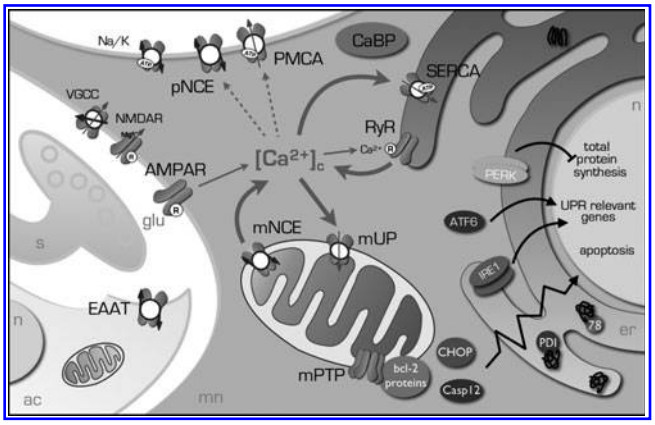


**FIG. 4. Interplay of α-amino-3-hydroxy-5-methyl-4-isoxazole propinate receptor (AMPAR) activity, mitochondrial and endoplasmic reticulum (ER) calcium cycling, and ER stress with the unfolded protein response (UPR).** Calcium shuttling forms a close functional link of mitochondria and the ER. A chronic toxic shift of calcium from the ER to mitochondria with exhaustion of the UPR leads to mitochondrial calcium overload and dysfunction with ROS increase, ER depletion with accumulation of misfolded proteins, and, eventually, cell death.

cells and in brain slices of adult G93A-SOD1 mice, the mitochondrial calcium content is reduced (260, 261). A dysfunction of the mitochondrial calcium storage can, thus, cause a disturbance of the ER calcium refilling, a reduction of ER calcium content, and an accumulation of unfolded proteins, which induces the UPR as a specific response to ER stress [for a review (214)].

The accumulation of unfolded proteins is detected by stress sensors, such as iron regulatory element 1 (IRE1), the double-stranded RNA-activated protein kinase such as ER kinase (PERK), and the activating transcription factor 6 (ATF6), which are released in ER stress conditions. ER stress triggers the oligomerization of IRE1, which leads to the splicing of XBP-1 mRNA. Spliced XBP-1 mRNA translocates to the nucleus and controls genes that are related to protein quality control, protein folding, components of the ER-associated protein degradation pathway, and the genes required for lipid synthesis [for a review (277)]. An increased amount of IRE1 and its phosphorylated form was reported in post-mortem sALS spinal cord and in G93A-SOD1transgenic mice. Accordingly, the amount of spliced, not of non-spliced, XBP-1 mRNA is increased in the spinal cord of symptomatic G93A-SOD1 mice (291) and in G85R-SOD1 transfected Neuro2a cells (405). Against expectations, the knock-down of the UPR sensor pathway by IRE1 and XBP-1 short hairpin RNA (shRNA) in G93A-SOD1 and G85R-SOD1 transfected NSC-34 cells decreases SOD1 aggregates and increases survival, possibly by the up-regulation of autophagy. Female G86R-SOD1 transgenic mice crossbred with XBP-1Nes−/− mice, which exhibit a XBP-1 deletion in the nervous system, have an increased lifespan, while their male counterparts do not (236); interestingly, human males are also more susceptible to ALS. This unexpected result was interpreted as a compensatory increase in autophagy, a dysfunction of which has been implicated in the pathogenesis of ALS (167). An activation of the PERK sensor pathway in ALS is indicated by increased amounts of PERK/p-PERK (20, 21, 392, 405, 463) and eIF2α/p-eIF2α (392, 405, 463) in G93A-SOD1 mice and Neuro2a cells expressing mutant SOD1. In human sALS post-mortem tissue, the PERK pathway is also activated (21, 236, 250), indicating a general disease-related mechanism. The experimental drug salubrinal inhibits the dephosphorylation of p-eIF2α, which results in a higher amount of active p-eIF2α, repression of protein translation, activation of salvage pathways, and low protein load (57). In Neuro2a cells expressing mutant SOD1, salubrinal treatment inhibits the SOD1 aggregation process, thereby decreasing cell death (405). G93A-SOD1 mice treated since the time of UPR initiation displayed an improvement in muscle force and lifespan (463). However, in this study, salubrinal treatment was applied before symptom onset (and, thus, in a condition untranslatable to patients), and proof of efficacy in treatment after symptom onset is still lacking.

ATF6 as the third ER stress sensor is transported to the Golgi apparatus, and sequentially cleaved. The cytosolic domain of ATF6 activates XBP-1, Grp78, and the CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) after nuclear translocation. ATF6 levels are elevated in G93A-SOD1 mice and human sALS samples (20, 21). Only cleaved ATF6, but not full-length ATF6, is increased in spinal cord G93A-SOD1 and G85R-SOD1 mice (291) and in Neuro2a cells expressing mutant SOD1 (405). Finally, ATF6 knock-down increases SOD1 aggregation in NSC-34 cells expressing mutant SOD1 (236).

*c. TDP-43 and VAPB in ER stress.* TDP-43 regulates the transcription, splicing, and stability of mRNA (63). When human mutant A315T TDP-43 is overexpressed in mice, a progressive ALS-like neurodegenerative disease ensues (577), whereas homozygous mice overexpressing wild-type human TDP-43 show a mild phenotype (584, 590). Interestingly, human sALS anterior horn cells with a nuclear translocation of TDP-43 display a fragmentation of the Golgi apparatus (181).

The transfection of NSC34 cells with human wild-type TDP-43 causes ER stress with increased CHOP expression and activation of the ER stress-responsive element (ERSE, a promoter containing an XBP-1- and an ATF6-binding motif). The ER stress-related caspase 12 seems to be involved in the cleavage of TDP-43 into C-terminal fragments, and the activation of apoptosis *via* Bcl-2 proteins is demonstrated by elevated Bim and decreased antiapoptotic bcl-XL. The expression of Bcl-2 itself seems not to be affected, but the amount of Bcl-2 mRNA is decreased (510). Thus, TDP-43 in its native form is directly involved in the ER stress response and cell death pathways, which can be exacerbated by mutations.

In contrast to TDP-43, VAPB is usually ER resident. VAPB maintains ER structure, protein transport, lipid metabolism, and the UPR. P56S and T46I mutant VAPB aggregate and lose ER localization (86, 398, 520). Both wild-type and P56S-VAPB interact with ATF6 and reduce the ATF6/XBP-1 dependent transcription with the mutant form inhibiting more strongly (199). In a viral vector-based *in vitro* model, the expression of both wild-type and P56S-VAPB in mouse primary MNs induces the same set of alterations, although with a different kinetic behavior; the UPR is activated and results in increased cell death that is prevented by treatment with salubrinal.

Since the reduction of intracellular $Ca^{2+}$, application of dantrolene (a blocker of ryanodine receptors), treatment with NBQX (an AMPA/KA antagonist), and inhibition of the ER-associated caspase cascade have a beneficial effect on the survival of MNs, VABP toxicity is likely mediated by a $Ca^{2+}$-dependent ER-associated mechanism (318).

Thus, ER stress and the activation of the UPR are common in the pathology of ALS. This may be due to the fact that a number of pathological processes converge to ER stress and the UPR activation, which appear to be quickly exhausted in MNs. The ER closely interacts with mitochondria and cytoplasmic calcium signals through the ERMCC, which senses energy demand and tightly couples energy production with protein folding (Fig. 4). Therefore, ERMCC disturbances induce UPR and vice versa. Since a persistent ERMCC dysregulation is a key pathway resulting in the accumulation of misfolded proteins, failing UPR and MN death, therapeutic drugs aiming at stabilizing the ERMCC, reducing ER stress, and supporting the UPR may be effective in ALS.

3. Nucleus. As discussed in the next few paragraphs, mutations in FUS/TLS and TDP43 possibly impact nuclear functions of the two proteins (*e.g.*, splicing regulation) that are important for MN viability. However, the possibility that mutations in SOD1 may affect a nuclear activity that is usually exerted by this enzyme has been already explored. Indeed, wild-type SOD1 also displays a nuclear localization (79, 109), and data suggest that this fraction of SOD1 protects nuclear DNA against a superoxide which is generated in or near the nucleus (6, 48, 173, 252). In an interesting article by the group of Poletti, it has been shown that while in wtSOD1 mice, motoneurons display an intense cytoplasmic and nuclear staining for SOD1, the majority of MNs in G93A-SOD1 transgenic mice show a predominant SOD1 labeling into the cytoplasm, leaving the nucleus unstained (462). Similar results were obtained in transfected mouse motoneuronal NSC34 cells. In this context, the cells overexpressing mutant SOD1 show a higher DNA damage compared with the wtSOD1-expressing cells when subjected to oxidative stress by $H_2O_2$, possibly because of a loss of nuclear protection.

In a different study exploiting human neuroblastoma cells stably overexpressing low levels of wild-type and mutated human SOD1, however, mutant SOD1 was found to be present in the nucleus in association with DNA; in this situation, the mutant enzyme seems to induce DNA damage and trigger the apoptotic response by activating p53 (30), suggesting a gained nuclear toxicity. Similarly, in transgenic mice overexpressing wt, G37R, or G93A human SOD1s, the nuclear accumulation of these proteins was observed, with only mutant forms inducing alterations of the survival motor neuron protein (SMN) complex in the nuclei of MNs (192).

The reasons for these discrepancies are unclear, but these data overall suggest that the nucleus is a previously unrecognized target of mutant SOD1 neurotoxicity in ALS that deserves further investigation.

## IV. RNA Dysmetabolism and ALS

Motor neuron diseases (MNDs), such as ALS, SMA, or lethal congenital contracture syndrome 1 (LCCS1), are a group of diseases that differ in transmission, genetic determinants, or in disease onset and durations (107, 344, 400). Still, they share several clinical and cell biological features: It appears, in fact, that damage to NMJs occurs early in the disease course, possibly followed by a dying-back mechanism of axonal degeneration and cell death. Most importantly, several of the recently identified genetic factors in MNDs (TDP-43 and FUS/TLS, SMN, and GLE1, for ALS, SMA, and LCCS1, respectively) encode proteins with an established role in RNA metabolism. Thus, RNA metabolism seems to be a unifying theme in MNDs, and appears to be the vulnerable metabolism in MNs. Since RNA metabolism comprises a large number of different processes, such as pre-mRNA splicing, mRNA transport, translational regulation, or mRNA decay, the precise RNA pathway that is affected in each of these diseases is still unclear, and, in the case of ALS, it is not completely clear whether defects in RNA processing have a causative, direct role in the pathogenesis, although the recent identified repeats in the gene *C9ORF7* in a large number of fALS cases strongly suggest that this is the case. Due to all these reasons, the identification of the precise RNA-related mechanisms underlying these defects has, thus, become the new frontier for researchers in MNDs, in general, and in ALS, in particular.

A brief overview on the structural and functional features of TDP-43 and FUS/TLS is provided in the next few paragraphs, while up-to-date details can be found in recently published reviews (63, 116, 310).

### *A. Structure and function of FUS and TDP-43*

Both TDP-43 and FUS/TLS are multifunctional hnRNPs that share a considerable structural homology and, probably, also functional overlapping (Fig. 5). FUS/TLS (also known as hnRNP-P2) is a 75 kDa nuclear protein of 526 amino acids, containing an RNA-recognition motif (RRM), a glycine-rich domain, two arginine, glycine, glycine domains (RGG), and nuclear localization and export sequences (NLS and NES) that drive the shuttling of FUS/TLS between the nucleus and the cytosol. Differently from TDP-43, it also contains a glutamine, glycine, serine, and tyrosine-rich domain at the N-terminal and a zinc finger motif, which is surrounded by the two RGG domains (140, 249). In 1993, FUS/TLS was first identified as a proto-oncogene that causes liposarcoma due to a chromosomal translocation (112, 435) which gives rise to a fusion (proto-onco) gene along with CHOP in human myxoid liposarcomas (112). Subsequent studies have shown that FUS/TLS is a prototypical member of the family of structurally similar proteins, the TET or FET family, including the Ewing's sarcoma protein (EWS), the TATA-binding protein-associated factor TAF15 (also known as TAFII68), and the closely related *Drosophila* cabeza/SARFH, all of which bind to RNA, single-stranded DNA, and also double-stranded DNA (516). A sequence specificity for RNA binding to FUS/TLS has been initially identified, in GGUG sequences in RNA targets (328). Now that a complete picture of the RNA-binding partners of FUS/TLS has been provided (240), it appears, however, that a significant consensus sequence for FUS is lacking, while an AU-rich stem-loop in the RNA structure might have a role in recognition.

Defining the biological functions of FUS protein is still difficult, although it is already clear that FUS/TLS is involved in many aspects of RNA regulation (Fig. 6). FUS/TLS affects transcriptional regulation, by interacting with transcription factors such as Spi-1/PU.1, YB-1, and NF-kB (221, 539), as well

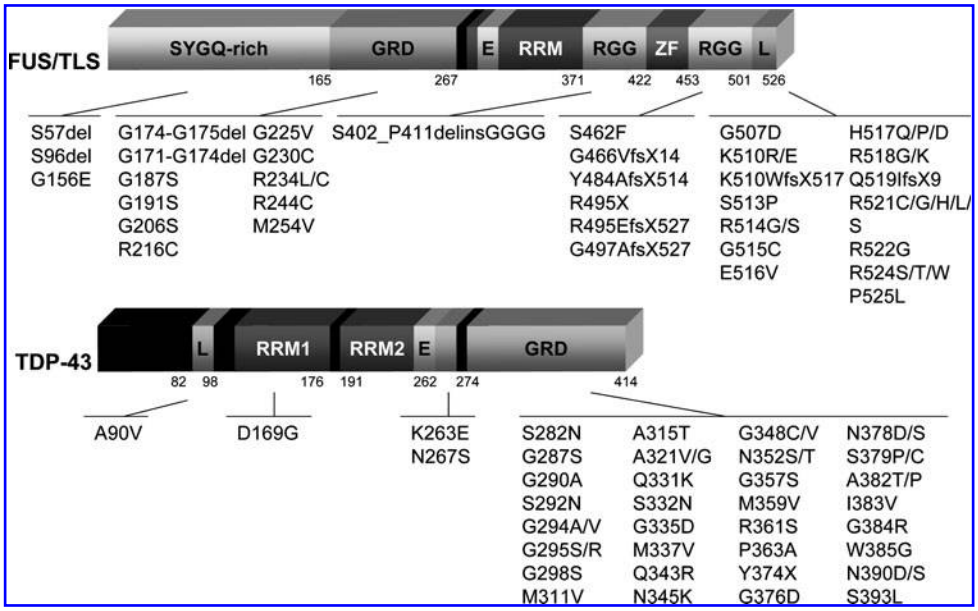


**FIG. 5. FUS/TLS and TDP-43 domains and mutations in ALS and frontotemporal lobar degeneration (FTLD) patients.** FUS/TLS is a 526-amino acid protein with several functional domains: an SYGQ-rich domain, with transcriptional activity; a glycine-rich domain (GRD) for protein-protein interaction; a nuclear export signal (E) for the export from the nucleus to the cytoplasm; a RNA recognition motif (RRM) for the binding to RNA; an arginine, glycine, glycine-rich region (RGG) for protein-protein interaction; a zinc-finger domain (ZF) for nucleic acid binding; and a nuclear localization signal (L) for the import from the cytoplasm to the nucleus. To date, 47 mutations have been described in sporadic ALS, familial ALS, and in rare FTLD patients: they are missense mutations, deletions, or in-frame insertions and truncations. TDP-43 is a 414 aa protein with several functional domains: L, nuclear localization signal, for the import from the cytoplasm to the nucleus; RRM1, for the binding of single-stranded RNA; RRM2, for chromatin organization and TDP-43 dimerization; E, a nuclear export signal; and GRD, a glycine-rich domain, at the C-terminal, important for protein-protein interactions, which also plays a role in alternative splicing. To date, 44 different TARDP mutations have been associated with ALS and FTLD, nearly all clustered in the extremely conserved GRD. All of the ALS-linked TDP-43 mutations are missense mutations except for one truncating mutation (Y374X).

as several nuclear hormone receptors (426), and by regulating the activity of RNA polymerase II and III (45, 517, 596). It also affects splicing regulation, by associating with transcription-splicing complexes and splicing factors (310). A role for FUS/TLS in the subcellular localization of mRNAs is supported by the observations that FUS/TLS is a part of an RNA granule that was isolated as a binding partner of KIF5, a member of the kinesin superfamily of molecular motors that target mRNAs to distinct cellular compartments (276). Moreover, FUS/TLS associates to RNA granules that are actively transported to dendritic spines on GluR5 activation (179), and it also facilitates the transport of mRNAs encoding for either $\beta$-actin or the actin-stabilizing protein Ndl-L to dendritic spines (180), thus suggesting a role for FUS/TLS in the regulation of spine morphology. Evidence suggests that FUS/TLS participates in the control of mRNA translation, as it assembles into RNA SGs, cytoplasmic foci consisting of mRNA and RNP complexes that are formed to repress translation under conditions of stress (12). Finally, the role of FUS/TLS in the maintenance of genomic integrity and in DNA double-strand break repair has been put forward by several sets of data (190, 238, 307, 574).

In 1995, the TDP-43 protein was first identified as a 43-kDa cellular factor binding the TAR regulatory sequence of LTR in the HIV-1 virus genome (hence the name of the gene, TAR DNA-binding protein, TARDBP) (412). The TARDBP gene (six exons) is located on chromosome 1 (1p36.22) and encodes for a 414-amino acid nuclear protein that contains two RRMs, a C-terminal glycine-rich region (GRR). TDP-43 has an NES and an NLS, enabling it to shuttle between the nucleus and the cytoplasm, potentially transporting bound mRNAs (28). Similar to FUS/TLS, it is well established that TDP-43 is involved in transcription regulation, as it is able to represses transcription by binding the DNA regulatory elements in the HIV-1 viral genome and in the promoter of the mouse SP-10 gene, and to associate with actively transcribed genes (Fig. 6). TDP-43 is associated with Drosha (210), an enzyme that is involved in the first step of miRNA maturation, and TDP-43 down-regulation affects the expression levels of miRNA let-7b and miR-663, which are capable of binding directly to TDP-43 (66). TDP-43 interacts with several proteins of the translation machinery (178), and co-localizes with the postsynaptic protein PSD-95 in RNA granules, along with Fragile X Mental Retardation Protein and Staufen 1, two RNA-binding proteins that are known to regulate mRNA transport and local translation in neurons (574), thus suggesting the role of TDP-43 in synaptic formation and neuronal plasticity. Interestingly, *Drosophila* lacking the TDP-43 homolog (TBPH) exhibit locomotive alterations and structural defects at the NMJs, thus reproducing some aspects of ALS pathology (159), and this phenotype is due to reduced protein levels in the microtubule-associated protein futsch/MAP1B, whose mRNA binds to and is stabilized by TDP-43 (200). TDP-43 also binds the low-molecular-weight NFL mRNA, and this binding was

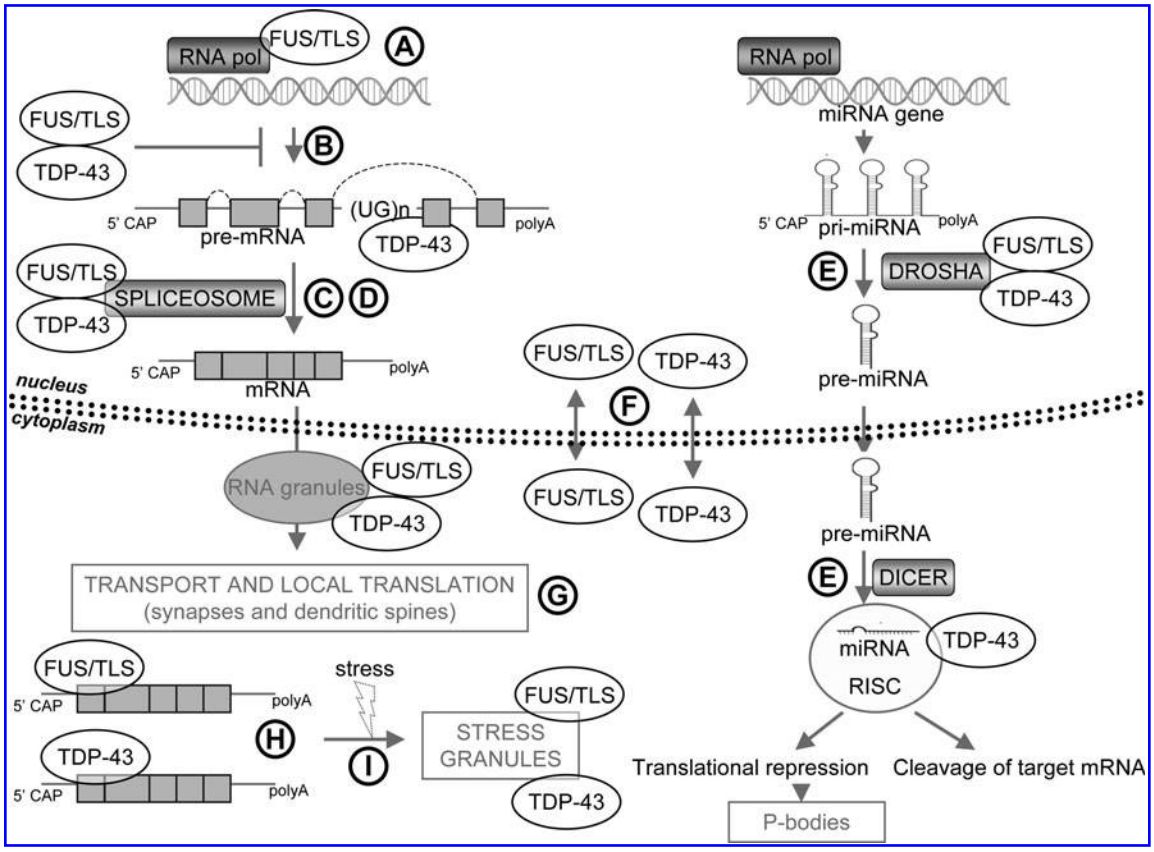


**FIG. 6. Involvement of FUS/TLS and transactive response DNA-binding protein (TDP-43) in RNA metabolism.** FUS/TLS and TDP-43 are involved in various steps of RNA metabolism, such as **(A)** Transcription: FUS/TLS associates with the transcriptional machinery and transcriptional regulators, thus influencing transcription initiation and promoter selection by RNA polymerase. **(B)** Transcriptional repression: FUS/TLS represses the transcription mediated by RNA polymerase III, while TDP-43 is known to repress HIV-1 and SP-10 gene transcription. **(C)** Splicing: FUS/TLS binds indirectly with the 5′ splice sites and directly to the 3′ splice sites of mRNA, and it also interacts with various splicing factors. TDP-43 may play a positive role in exon inclusion during the splicing regulation of survival of motor neuron (SMN)1/2 exon 7. TDP-43 also binds to splicing factors such as SC-35 and hnRNP A2. **(D)** Alternative splicing: The two proteins also have effects on the alternative splicing of some proteins. **(E)** miRNA processing: Both TDP-43 and FUS/TLS have been found in association with DROSHA complex that processes pri-miRNA into pre-miRNA, and TDP-43 was also found in association with proteins which participate in the cleavage of pre-miRNA mediated by Dicer into the cytoplasm. **(F)** Nucleo-cytoplasmic shuttling: TDP-43 and FUS/TLS shuttle between the nucleus and the cytoplasm. **(G)** Transport and local translation: in neurons, TDP-43 and FUS/TLS have been found in RNA granules translocating to the axonal terminal and to dendritic spines on GluR5 activation. **(H)** Translation: FUS/TLS and TDP-43 also participate in the control of mRNA translation. **(I)** Stress granules: TDP-43 and the mutant forms of FUS/TLS are recruited to stress granules on different environmental stressors.

demonstrated as stabilizing the NFL transcript (499, 558, 559). TDP-43 is also capable of controlling its own expression through a negative feedback loop by binding to 3′ UTR sequences in its own mRNA and promoting RNA instability, thus indicating the role of TDP-43 in the control of RNA stability, and also suggesting that disease-associated TDP-43 mutants disrupt TDP-43 self-regulation, thus contributing to pathogenesis (23). Finally, the initial evidence suggesting that the biological function of TDP-43 might be related to alternative splicing regulation, as indicated by its ability to bind to splicing-regulating factors, such as SC-35 and hnRNP A2, and to influence the splicing patterns of specific target mRNAs, such as mRNA for CFTR, apolipoprotein A-II, and SMN (64), has found a remarkable confirmation by recent experiments of crosslinking and immunoprecipitation (known as CLIP), followed by high-throughput sequencing of the RNAs bound to TDP43 (425, 527). Indeed, a large number of TDP-43 RNA binding sites has been identified (ranging from 30,000 to 100,000, depending on the particular CLIP technique used), and multiple RNA targets of TDP-43 have been also characterized in two other studies (472, 589). From the analysis of the results obtained from different experimental samples, including postmortem brain samples from people with FTLD, as well as mouse brains and cultured human neuroblastoma with down-regulated TDP-43, at least two major conclusions can be drawn: (i) TDP-43 has an important function in splicing regulation: This is clearly indicated by the observation that most of the TDP-43 binding sites lie in intronic positions (in long clusters of UG-rich sequences), and by alterations in the splicing pattern of hundreds of transcripts, including some that had been already reported; (ii) TDP-43 affects the expression levels of several transcripts, with many likely playing important roles in neuronal activity and neurodegeneration, including the mRNA for FUS/TLS. Thus, although these studies neither

indicate the specific RNA targets that are relevant for ALS, nor provide evidence for a particular disease mechanism, they have provided an important framework for understanding how TDP-43 and RNA dys-metabolism contribute to ALS.

### *B. FUS/TLS and TDP-43 and the pathogenesis of ALS*

The toxicity of mutant FUS/TLS and TDP-43 proteins may be related to their altered intracellular localization, a feature which marks tissues from patients and that is reproduced when mutant TDP-43 and FUS/TLS are overexpressed in cultured cells (Fig. 1), indicating that mislocalization is a common trait of mutated proteins, and it is strikingly in contrast with the predominant nuclear localization of the two proteins in normal cells (308, 395, 396, 549), which indeed reflects at least a fraction of their physiological function. Moreover, redistribution of the mutated proteins in the cytosol seems to be paralleled by a depletion of the same proteins from the nucleus (17, 308, 345, 395, 396, 549) and, thus, the loss of a nuclear function can be imagined as the mechanism leading to pathology (loss of function). However, the accumulation of a nuclear protein in the cytosol, particularly in an aggregated form, can endow this protein with a new, toxic function (gain of function). Whether ALS linked to TDP-43 or FUS/TLS results from a loss or a gain of functions, or whether the two features are simultaneously shared, is unknown, and, in any case, the nature of the disease mechanisms remains unclear (Fig. 7).

Given the multiple roles of FUS and TDP-43 in RNA regulation, it is readily expected that some of the functions just outlined are affected by pathogenic mutations, and that these alterations are more or less directly responsible for the specific demise of MNs in ALS. However, at the moment, detailed disease mechanisms related to specific RNA-regulated pathways have not been described.

Most of the functions described for FUS/TLS and TDP-43 controlling RNA metabolism deal with a cytosolic localization of the two proteins; however, in a physiological condition, both FUS/TLS and TDP-43 exhibit a predominant nuclear localization. Indeed, nucleo-cytoplasmic shuttling is testified by the presence of canonical NES/NLS in TDP-43 and a nonclassical NLS in the C-terminal region of FUS/TLS (a.a. 514–526) that has been recently confirmed to mediate nuclear import through the nuclear import receptor, transportin (140). Since nuclear depletion of ALS-linked mutants of both TDP/43 and FUS/TLS is a hallmark of pathology in cell and animal models of the disease, as well as in patients (345), it is simple to imagine that mutations affect the ability of the two proteins to move between the nucleus and the cytosol, thus impinging on an important mechanism whereby FUS/TLS and TDP-43

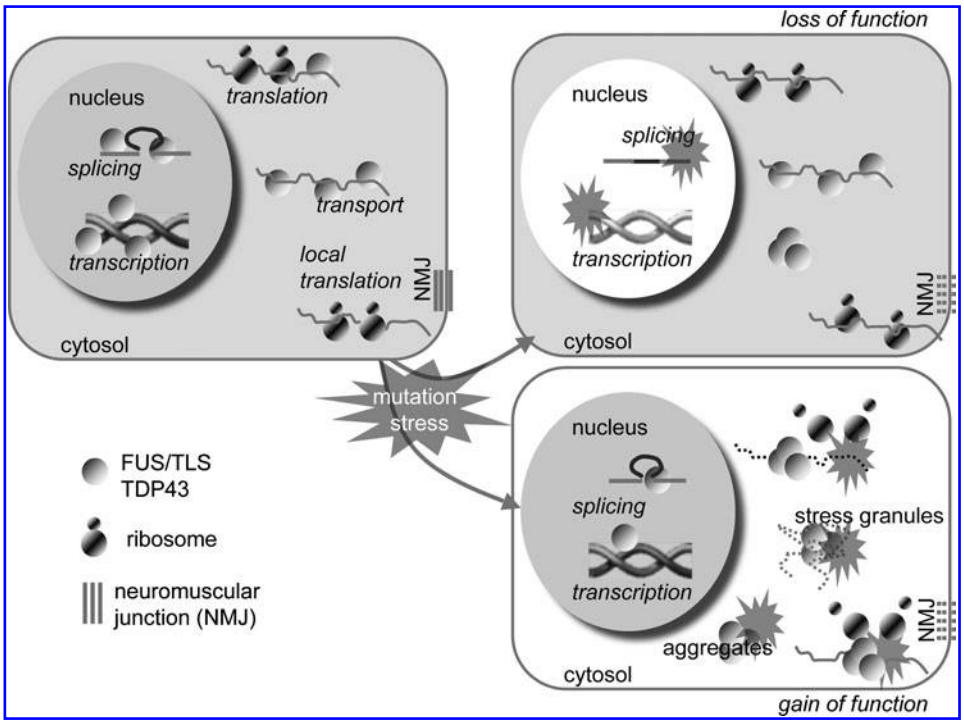


**FIG. 7. The gain-and-loss-of-function hypothesis for the toxicity of FUS/TLS and TDP-43 in motor neurons.** Both TDP-43 and FUS/TLS are mainly localized in cell nuclei, where they control gene transcription and pre-mRNA splicing. By shuttling between the nucleus and the cytosol, TDP-43 and FUS/TLS might participate in the control of mRNA translation, transport, and local translation at the NMJ. In the presence of mutations, as well as after stress, the two proteins accumulate in the cytosol. As a consequence, the supply of nuclear TDP-43 and FUS/TLS is affected, and the splicing and/or translation of genes controlling NMJ stability and motoneuronal viability is impaired or lost (loss-of-function mechanism). As an alternative, the FUS/TLS and TDP-43 accumulated in the cytosol might acquire a toxic activity that is detrimental to the NMJ (gain of function). This property might descend from the aggregation of the two proteins into cytoplasmic inclusions, from accumulation into stress granules, and/or from the alteration in the activity of complexes controlling mRNA translation and transport. Evidence for the existence of either one mechanism or the other has been reported, but it cannot be excluded that they may act simultaneously.

function is accomplished. This seems most likely for FUS/TLS, given that the majority of ALS-related mutations described to date cluster in the NLS of the protein. On the contrary, robust evidence sustaining the hypothesis that TDP-43 mutations associated to the disease directly affect the NLS, and, thus, the nuclear import of TDP-43, is still lacking; nor the position of ALS-related mutations give support to this hypothesis, given that almost all mutations are located in the C-terminal GRR, which mediates the interaction with other hnRNPs, and none of the TDP-43 mutations so far described affect the N-terminal NLS. Therefore, other properties of TDP-43 (and of FUS/TLS as well) might be affected by mutations, leading to the accumulation of the protein in the cytoplasm and the consequent depletion from the nucleus.

TDP-43 and FUS/TLS were found to be recruited into SGs in conditions of stress (12, 52, 97, 140, 178, 186, 377, 397) and, as mentioned earlier, this is an important clue to the concept that both proteins might control SG formation/functions, and, consequently, mRNA translation and stability (62). These observations suggest that ALS-causing mutants might affect the normal response to stress that includes translation inhibition through SG formation. In other words, is the FUS/TLS or TDP-43–mediated control of stress response affected by pathological mutations? Although it has been shown that wild-type FUS/TLS is able to associate to SGs in stress conditions, indeed fALS-linked mutations strongly enhance FUS/TLS association with SGs (140), thus indicating that MN vulnerability in ALS could be related, in part, to altered adaptation to cellular stresses. Based on the effect of mutation on FUS/TLS nuclear localization and SG formation, Dormann *et al.* have proposed a two-hit model of FUS/TLS pathology. According to this model, a mutation—the first hit—supports an increased cytosoloic localization of FUS/TLS, which, however, is not sufficient for the formation of FUS-containing granules unless a stress—second hit—causes the formation of FUS-containing SGs. Since SG marker proteins also co-deposit with FUS in the brains of fALS and FTLD-FUS patients (140), a chronic accumulation of mutant FUS/TLS in SGs might prelude to the pathological formation of inclusions. It will be interesting to test this model in other experimental settings, and, *in vivo*, in animal models of the pathology, and to check whether a multiple-hit model can also be extended to TDP-43 pathology.

TDP-43 and FUS/TLS have been shown to physically interact, and to associate as parts of high-molecular-weight complexes (294, 303, 336). Moreover, the depletion of TDP-43 leads to the down-regulation of FUS mRNA (169, 425). Thus, the two proteins seem to act in the same pathway. This hypothesis has now found a strong confirmation from experiments in the zebrafish model, where wild-type FUS was shown to rescue the motor phenotype induced by TDP-43 knock-down, but not vice versa, indicating that FUS/TLS lies downstream of TDP-43 (270). Interestingly, SOD1 rescues the motor phenotypes in zebrafish from neither FUS/TLS nor TDP-43 down-regulation; nor are FUS/TLS and TDP-43 able to rescue the motor deficits induced in zebrafish by mutSOD1 overexpression (270), suggesting a different pathogenic mechanism for mutant SOD1.

A great number of animal models with a genetically modified expression of TDP-43 have been produced, while less have been described to date for FUS/TLS, although it is much likely that this number will increase exponentially in the near future. They are summarized in Table 4 (TDP-43) and Table 5 (FUS/TLS) and have been very accurately reviewed in the recent past (116, 266). Almost all of them can reproduce some of the neurodegenerative phenotypes observed in humans, including, in the case of TDP-43, cytoplasmic accumulation and nuclear clearance of the protein, and, thus, confirming the neurodegenerative potential of the two proteins; however, none of them completely recapitulates the human pathology. As a remarkable example, cytoplasmic ubiquitinated TDP-43 inclusions, which are very common in the neurons of ALS patients, do not represent a major histopathological feature of rodent models, thus arguing against the role of cytoplasmic inclusion of TDP-43 in the pathology, or as an alternative, against the ability of these animals to model human ALS. However, they will certainly help in addressing the principal issues of TDP-43 and FUS/TLS pathology related to the role of mis-localization and aggregation of the proteins, the nature of RNA pathways and RNA targets that are relevant for the disease, and the contribution of different tissues to the selective degeneration of MNs, a topic that has changed our view of this disease to a great extent.

## V. Non-Cell-Autonomous Death of MNs

Despite its classical clinical description as an MND, ALS does not purely affect MNs. A number of studies have addressed the contribution of non-neuronal cells to the pathogenesis in models based on the expression of mutant SOD1 (322) and already in the years 2003–2006, genetic and chimeric mice studies using SOD1 models provided clear evidence of a non-cell autonomous death of MNs [(Fig. 8) (49)], where ALS seems to be a two-phase process, in which onset and progression seem to depend on the damage within different cell types.

### *A. Neurons and glia*

Transgenic mice that express mutant SOD1 only in MNs do not show neurodegeneration (337), unless the level of mutant SOD1 is highly increased in MNs, or also expressed in interneurons (258, 570). The expression of mutant SOD1 in neurons determines the timing of onset of disease and of an early phase of disease progression, while a diminished expression of the mutant enzyme in microglial cells slows disease progression (50). Furthermore, silencing mutant SOD1 expression in neurons through an injection of a lentiviral vector into various muscle groups in the presymptomatic stage results in a marked delay of onset, without a significant variation in disease duration (437). This concept has been further supported by more recent studies that provide evidence on the effect of the expression of mutant SOD1 purely in neurons under control of the neuron-specific promoter Thy1.2 (258). These animals show signs of neurodegeneration only very late in their life, with an onset between 1 and 2 years of age, compared with 100–150 days of "classical" G93A-SOD1 mice with ubiquitous expression of the mutant enzyme, and the process is extremely slow, never reaching the level obtained in G93A-SOD1 mice.

In a series of elegant articles by Don Cleveland's group and collaborators, the contribution of non-neuronal cells to ALS onset and progression was estimated using either chimeric mice or mice with modulated cell-specific expression of mutant SOD1. The expression of high levels of mutant SOD1 only in MNs and oligodendrocytes, in a cellular environment

Table 4. Transactive Response DNA-Binding Protein-43 Transgenic Animal Models

| *Species* | *Transgene* | *Promoter/tissue expression* | *Phenotype* | *References* |
|---|---|---|---|---|
| *Caenorhabditis elegans* | WT | Pan neuronal | Nuclear accumulation of the protein<br>Abnormal NMJ. Moderate motor deficits | (19, 334, 606) |
| | ΔRRM1, ΔRRM2, ΔC-term | Pan neuronal | Granular staining pattern<br>Normal phenotype | (19) |
| | G290A, A315T, M337V | Pan neuronal | Nuclear TDP-43 aggregates<br>Neurodegeneration<br>Severe motor dysfunction | (334, 606) |
| *Drosophila* | WT | Eye | Retinal degeneration | (150, 320, 333, 367) |
| | | MNs | Motor dysfunction. Paralysis<br>Reduced lifespan | (224) |
| | | | Cytoplasmic TDP-43 aggregates. Axon swelling reduction in axon branches and bouton numbers. Loss of MNs and functional deficits | (333) |
| | | | Reduced movements and reduced lifespan | (367) |
| | | | Severe motor deficits. Paralysis | (557) |
| | | Pan neuronal | Lethal | (557) |
| | ΔNLS | Eye | Severe retinal degeneration | (367, 447) |
| | | Neuronal/glia/ muscle | Pupal lethality | (367) |
| | ΔNES | Eye | Mild retinal degeneration | (367) |
| | | | Normal | (447) |
| | | Neurons | Early death | (367) |
| | Q331K | MNs | Severe motor deficits | (150) |
| | M337V | Eye | Degeneration of photoreceptor cells | (447) |
| | G287S, A315T G348C, A382T N390D F147L/ F149L | MNs | Progressive motor deficits and paralysis | (557) |
| Zebrafish | WT, A315T | Embryos | Axonal shortening<br>Motor deficits | (271, 313) |
| | A382T, G348C | Embryos | Premature and excessive branching<br>Shorter MNs axons. Swimming deficits | (271) |
| Mouse | WT | mPrp/neurons and spinal cord | Het: Normal phenotype. Mild astrogliosis | (492, 590) |
| | | | Hom: Astrogliosis in spinal cord<br>Axon degeneration without loss of MNs<br>Cytoplasmic phospho-TDP-43 aggregates<br>Mitochondrial aggregates | (590) |
| | | Thy1/Neurons of the central nervous system | Het: Normal | (584) |
| | | | Het: Retardation of development related to copy number of transgene. Abnormal NMJs. Reduction in large-caliber motor axons. Mitochondrial aggregation. Intranuclear inclusions of TDP-43 | (473) |
| | | | Hom: Degeneration of cortical and spinal MNs (ALS phenotype) and non-motor cortical and subcortical neurons (FTLD phenotype) | (584) |
| | | CaMKII/ Forebrain | Hom: Learning and memory deficits. Progressive motor dysfunction. Gliosis. Reduced volume of hippocampus. Cytoplasmic Ubiquitin-TDP-43 positive inclusions | (534) |
| | | CaMKII-tTA/ forebrain | Het: Neuron loss in dentate gyrus. Activation of astrocytes and microglia in cortex and hippocampus. Predominantly nuclear TDP-43 localization | (246) |
| | | CAG/ubiquitous | Age-related moderate loss of cortical neurons | (521) |
| | ΔNLS | CaMKII-tTA/ forebrain | Het: Neuron loss in cortex and hippocampus<br>Corticospinal tract degeneration. Spasticity (FTLD and PLS phenotype). Rare phosphorylated and ubiquitinated aggregates | (246) |

(*continued*)

Table 4. Continued

| Species | Transgene | Promoter/tissue expression | Phenotype | References |
|---|---|---|---|---|
| | A315T | mPrp/neurons and spinal cord | Het: Normal up to 3 months. Gait disturbance (3–4 months). Premature death. Astrogliosis Loss of MNs. Rare phospho-TDP-43 inclusions | (577) |
| | | | Het: Gliosis. Muscle atrophy Rare phospho-TDP-43 inclusions | (492) |
| | WT, G348C, A315T | Endogenous/ ubiquitous | Age-related impairment in learning and memory Motor dysfunction. Age-dependent astrogliosis and microgliosis. Cytoplasmic aggregates, neurofilaments abnormalities, and reduced axonal caliber for mutant TDP-43 | (513) |
| | M337V | CAG/ubiquitous | Early death in transgenic founders | (521) |
| Rat | WT | CAG-tTA/ ubiquitous | Normal | (614) |
| | M337V | CAG-tTA/ ubiquitous | Progressive degeneration of MNs denervation and atrophy of skeletal muscles Early death. Cytoplasmic localization of phospho-TDP-43. Rare cytoplasmic inclusions | (614) |

CaMKII, Ca-calmodulin-dependent kinase II; Het, heterozygous; Hom, homozygous; MN, motor neuron; mPRP, mouse prion promoter; NES, nuclear export signal; NLS, nuclear localization signal; NMJ, neuro muscular junction; PLS, primary lateral sclerosis; RRM, RNA recognition motif; Thy1, thymocyte differentiation antigen 1 promoter; tTA, inducible expression by tetracycline transactivator; Δ, nucleotide deletion.

containing variable numbers of nonmutant, non-MNs, delays the onset of MN degeneration, thus indicating that expression in cell types other than MN and oligodendrocytes accelerates onset (591). On the other hand, decreasing the expression of mutant SOD1 in astrocytes only does not affect onset, but delays microglial activation and slows disease progression (592). Astrocytes also contribute to ALS, as originally suggested by two studies showing that astrocytes expressing mutant SOD1 release factors are selectively toxic to MNs (3, 76, 135, 389) and very recently confirmed by the observation that astrocytes expressing the G93A mutation induce wild-type MN degeneration *in vivo* (415) and, thus, should be considered "deadly neighbors" of MNs (268).

A non-cell-autonomous glial effect has also been observed in a model for alsin-linked ALS, with a higher susceptibility of upper *versus* lower MNs (259).

Changes in inhibitory interneurons may also contribute to ALS. In spinal cords of not only classical low-copy G93A-SOD1 mice, but also mice with neuron-specific expression of G93A-SOD1, reduced interneuronal expression of glycine transporter 2 and glutamic acid decarboxylase and appearance of ubiquitinated inclusions were found in the ventral horns of symptomatic mice. Accordingly, it has been proposed that damage may spread from MNs to interneurons in an early phase of the disease, and, in turn, the degeneration of spinal inhibitory interneurons may affect MNs and contribute to disease progression (242).

A number of studies support the concepts that neuroinflammation is a pathological hallmark of ALS. As already suggested by early studies *in vitro* (166), neuronal injury depends on a cross-talk between MNs and microglia. Resting microglia from ALS transgenic mice show a diminished capacity to respond to tissue disturbances, thus having an initially reduced neuroprotective function (284, 457). At later stages, the activation of microglia at sites of neuronal injury becomes toxic. This cross-talk is mediated by the signals released from MNs that activate microglia. Activated microglia, in turn, switch from being anti-inflammatory and neuroprotective to being proinflammatory and neurotoxic, releasing ROS and pro-inflammatory cytokines and further enhancing MN injury (15).

Interestingly, evidence of inflammation has been reported not only in the mutant SOD1 mice, but also in studies on mice expressing wild-type (513, 592) or mutant (492, 513, 577) human TDP-43, which exhibit reactive gliosis accompanying axonal degeneration. Astrocytic and microglial activation is evoked in mice even when TDP-43 is transgenically overexpressed only in the forebrain (534) and when human TDP-43 is expressed without its NLS (246). Transgenic rats expressing mutant FUS/TLS also show degeneration of motor axons, and neuronal loss is accompanied by a marked glial reaction (243).

Beside inflammatory events concerning innate responses mediated by the activation of resident macrophages and microglia in the CNS, an adaptive immune response mediated by the infiltration of T lymphocytes and dendritic cells secreting macrophage-activating cytokines seems to contribute to MN death and the progression of ALS, as suggested by many studies in the postmortem spinal cord and the motor cortex of sALS and fALS patients (93). High levels of mRNA and protein belonging to the family of complement components are present in the spinal cord and the motor cortex from patients with rapid and slow sALS, along with higher levels of activated microglia, reactive astrocytes, dendritic cells, and CD8 (+) T cells (491).

A few studies on the immune system of sALS patients revealed significant changes in peripheral T cells and circulating monocytes (14), and recent evidence also supports a role for purinergic signaling in ALS (562).

In the G93A-SOD1 mouse model of ALS, the levels of natural killer T cells is dramatically increased, and T-cell distribution is altered in both lymphoid organs and the spinal

Table 5. Fused in Sarcoma/Translocated in Liposarcoma Protein Transgenic Animal Models

| Species | Transgene | Promoter/tissue expression | Phenotype | References |
|---|---|---|---|---|
| *Drosophila* | WT | Eyes | Depigmentation | (87) |
| | | | Mild ommatidial degeneration | (320) |
| | | MBs | Decreased size of MB | (87) |
| | | Neurons | Progressive axonal loss | |
| | | MNs | Reduced larval movements | (87) |
| | | | Normal | (320) |
| | | Pan neuronal | Normal | (320) |
| | R524S, P525L | Eyes | Severe ommatidial degeneration | (87) |
| | | MBs | Decreased size of MB | (87) |
| | | Neurons | Progressive axonal loss | |
| | | MNs | Severe MN damage and locomotive impairment | (87) |
| | R518K, R521C, R521H | Eyes | Disorganized ommatidia and loss of mechanosensory bristles | (320) |
| | | Pan neuronal | Pupal lethality | (320) |
| | | MNs | Morphological defects at the NMJ | (320) |
| | ΔNES, ΔNES/R518K, ΔNES/R521C | Eyes/MNs | Normal | (320) |
| Zebrafish | WT, H517Q, R521G, R495X | Embryos | Nuclear localization of WT and mut H517Q, R521G. Cytoplasmic localization of mut R495X | (52) |
| | R521H, R521C | Embryos | Shorter MN axons. Motor phenotype | (270) |
| Rat | WT | CAG-tTA/ubiquitous | Het: Loss of neurons in cortex and hippocampus. Age-related impairment of learning and memory | (243) |
| | R521C | CAG-tTA/ubiquitous | Het: Degeneration of motor axons. Neuronal loss in cortex and hippocampus. Astrogliosis in cortex and spinal cord. Loss of NMJs<br>Reduced learning and memory<br>Weight loss. Paralysis | (243) |

MBs, mushroom bodies.

cord, with pathological consequences in "non-affected" organs such as the liver (170). Complement activation products are elevated in G93A-SOD1 mice at an symptomatic stage in spinal cord astrocytes and MNs, and before the appearance of clinical symptoms at the NMJ (237). Innate immunity also plays a role in the peripheral nervous system of the G93A-SOD1 mice, where macrophage activation occurs presymptomatically, and activated macrophages infiltrate from the circulation in peripheral nerves (93).

Supporting the role of inflammation in ALS, granulocyte colony-stimulating factor (GCSF) attenuates inflammation in the CNS and the periphery and delays the progression of the disease in the G93A-SOD1 mice (424).

### B. Contribution of other cell types

Schwann cells may also play a role in the progression of the disease. Using a Cre/Lox system, mutant SOD1 was selectively silenced in these cells in a line of G37R-SOD1 mice and, quite unexpectedly, these mice have an unchanged onset and an early symptomatic phase than control mice, but survival is markedly decreased, indicating that expression of the mutant enzyme in Schwann cells is protective, rather than detrimental (340). In line with this view, the accumulation of the mutant SOD1 within Schwann cells is not pathological to spinal MNs or deleterious to the disease course in the transgenic ALS model mice (535). This may be linked to an artificially higher and protective level of SOD activity in these cells, and to the fact that Schwann cells secrete IGF-1, which is known as a positive factor in axon regeneration that has positive effects in two independent studies on ALS mice (139, 281). These two studies also underlie the concept that muscle cells also may play a role in the pathogenesis of ALS, a concept that has been widely discussed in the recent past. In fact, many studies in ALS animal models have shown that MN death starts with destruction of the NMJ and degeneration of the distal part of the axon (146) (Fig. 3). Retraction of motor axons from synaptic connections to the muscle seems to be among the earliest presymptomatic events in ALS. As demonstrated in an electrophysiological study on the G93A-SOD1 mice, alterations of central motor pathways are found to occur simultaneously to lower motoneuron dysfunction, well before functional abnormalities appear (350), and using MRI to monitor changes throughout the disease in the brain and skeletal muscle of the same mice, Marcuzzo *et al.* have shown that hind limb muscle atrophy precedes cerebral neuronal degeneration (352). Thus,

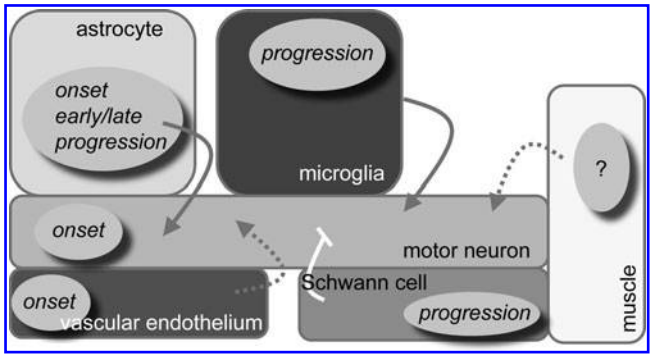


**FIG. 8. Non-cell autonomous degeneration of motor neurons.** In SOD1-linked mouse models of ALS, SOD1 expression in motor neurons is sufficient to cause the disease onset. However, surrounding cells, mostly affecting disease progression, strictly govern the pathology. In particular, decreasing SOD1 expression in microglia delays disease progression, while not only both early and late phases of progression, but also the onset, are delayed when SOD1 is reduced in astrocytes. SOD1 expression within muscle cells induces signs of ALS, while a partial suppression of its expression does not affect the disease course, thus leaving the exact contribution of muscle cells to the pathology unclear (*dotted arrow*). SOD1 expression in Schwann cells seems to confer neuroprotection, and the reduction of its expression mostly affects disease progression. SOD1 damage within vascular cells accumulates before motor neuron degeneration, indicating a role in disease initiation, but the removal of SOD1 from endothelial cells does not affect the disease course, thus leaving uncertain where this effect originates (*dotted arrow*).

MNs may be lost as a consequence of the "dying-back" phenomenon in ALS (117).

However, viral delivery of transcription-mediated siRNA that suppresses mutant SOD1 accumulation within muscle alone is insufficient to maintain grip strength, whereas delivery to both MNs and muscle is sufficient, and decreasing the level of mutant SOD1 in muscle alone does not affect the onset or survival (372). Nonetheless, skeletal muscle is a target of oxidative stress that is induced by mutant SOD1 (138), and muscle-restricted expression of a localized IGF-1 isoform in G93A-SOD1 mice not only maintains muscle integrity and stabilizes NMJs, but also reduces inflammation in the spinal cord and enhances motor neuronal survival, delaying the onset and progression of the disease (139). These effects are related to the ability of the muscle IGF-1 to modulate ubiquitin expression, caspase activity, CDK5, and the toxic p25 protein, thus allowing the maintenance of the muscle phenotype with a normal peripheral nerve and a greater number of myelinated axons (138). Furthermore, as a consequence of oxidative stress, Ras-related association with diabetes (Rad), an inhibitor of voltage-gated calcium channels, is up-regulated in the muscle in patients and in mice at asymptomatic stages (202, 222). Transgenic mice expressing wild-type-, G37R-, and G93A-SOD1 only in the skeletal muscle display neurologic and pathologic phenotypes that are consistent with ALS, including marked NMJ abnormalities (587). Further, muscle-specific mitochondrial uncoupling induces the deterioration of the NMJ, is a sign of denervation and MN pathology, and exacerbates the pathology of an ALS animal model (145). Overall, these results indicate that muscle dysfunction plays a primary role in ALS.

The systemic expression of three different mutant SOD1s also has the effect of disrupting the blood-spinal cord barrier in mice by reducing the levels of several proteins (zona occludens 1 [ZO-1], occludin [Ocln], and claudin-5 [Cldn5]) involved in the formation of tight junctions between endothelial cells (188, 189, 612). This endothelial damage results in microhemorrhages before overt MN degeneration and before the occurrence of the neurovascular inflammatory response, suggesting that this process may play a role in disease initiation (612, 613). Notably, the level of mRNAs coding for the ZO-1 and Ocln is decreased in postmortem spinal cord tissues from ALS patients (230).

On the whole, these studies indicate that each cell type (MNs, interneurons, glia, muscle, Schwann cells, endothelial cells, and even lymphocytes) contributes in a different way to the onset and progression of disease in the mutant SOD1 mice. Whether results from these studies may be generalized to patients remains to be established, especially in the light of published reports on the differential effects of cell-specific knock-down of two different mutant SOD1s (G85R *vs.* G37R) on MN death. Deletion from MNs and interneurons primarily delayed disease onset and early disease progression in both mice (567). However, G85R-SOD1 knock-down in microglia/macrophages induced a prolonged early and late disease phase while G37R-SOD1 knock-down in the same cells only affected late phase (571); and astrocyte knock-down of G85R-SOD1 in mice delayed disease onset, prolonged the early phase of disease progression, without affecting the late phase, and resulted in decreased microgliosis, decreased SOD1 aggregates, and the preservation of GLT-1 (EAAT2) transporter expression (568).

### C. Extracellular signals

The concept of non-cell autonomous death of MNs in ALS can be expanded by considering that damage within different cell types may be integrated by toxic signals exchanged by different cell types. Most of the chemical mediators identified so far are ROS or proinflammatory molecules (232). Mutant SOD1 may exert an extracellular toxic action as well. Misfolded SOD1 was detected in the CSF of fALS patients using antibodies specifically recognizing misfolded SOD1 species (603), in line with the concept that SOD1 is excreted from neurons (536). Secretion is mediated by interactions with chromogranin A and B (542), and the overexpression of chromogranin A in neurons accelerates the disease onset and gliosis in mutant SOD1 mice (154).

Misfolded extracellular mutant SOD1 not only seems to be caable of prion-like propagation in neuronal cells (387) but can also be toxic to non-neuronal cell-triggering microgliosis (542); in turn, activated microglia mediates MN injury (611). Furthermore, the extracellular mutant SOD1 may form membrane channels and induce depolarization and increases in intracellular calcium, as demonstrated in mouse neuroblastoma cells (10).

A clear demonstration of the role of extracellular SOD1 in ALS comes from the observation that vaccinations targeting extracellular SOD1 mutants are beneficial in transgenic mutant SOD1 mice (540). Extracellular SOD1 may also be relevant for the modulation of vascular tone, as wild-type SOD causes endothelium-dependent relaxation (383). In this light,

mutant SOD1 may be involved in ALS pathogenesis *via* blood-brain barrier disruption as mentioned earlier (612).

## VI. Therapeutic Approaches

### A. Pharmacological therapies in ALS/clinical update

Over the past 20 years, many ALS treatment trials have been underpowered or governed by insufficient disease markers (115), most likely because ALS is a very heterogenous disease in terms of age of onset, progression rate, and primarily affected regions. Bulbar onset with swallowing difficulties tends to cause earlier demise, whereas cervical onset leads to loss of arm function and diaphragm paralysis, thoracic onset due to loss of axial stability and respiratory support muscle weakness, and lumbar onset due to loss of ambulation. El Escorial criteria for inclusion into clinical trials may have been too exclusive, and have been revised (35). It appears that patients today live longer than they did a decade ago, which has largely been attributed to better care, the use of gastrostomy, and noninvasive ventilation (434). Currently, the only drug known to slow motoneuron degeneration in ALS is riluzole. It prolonged life in two large, international, well-designed clinical trials, albeit only by 2–3 months. Despite numerous investigations, it is unknown by which mechanism riluzole exerts its protective activity. It reduces glutamate release by a presynaptic mechanism, but has such a plethora of other plasmalemmal and intracellular effects that it has been termed a "dirty drug" (36, 40). Beyond riluzole, a whole range of drug classes has been tested but without demonstrable benefit for patients [for review see (4, 319, 532, 616)]. Recent and ongoing trials include ceftriaxone to reduce synaptic glutamate, arimoclomol to amplify HSP gene expression, immunization to remove misfolded proteins, patient-specific antisense oligonucleotides to reduce mutant SOD1, intracerebroventricular VEGF, safety tests for the application of embryonic and iPSCs, and skeletal muscle targets such as troponin activator, GDF-8 myostatin inhibitor, and Reticulon 4, the latter that promotes neurite growth (616). In a meta-analysis of past failure that translates positive results obtained in the mutant SOD1 mice into clinical practice, Benatar (37) suggested that "drugs such as minocycline and Cox-2 inhibitors with an anti-inflammatory mechanism of action, and antioxidative agents such as creatine or the manganese porphyrin AEOL-10150, appear to be the most promising for preventative and therapeutic trials respectively in patients with fALS." However, trials with minocycline (204) as well as a number of trials with antioxidants have failed so far. Obviously, this may be due either to our incomplete understanding of mechanisms operating in the disease, or to the choice of drugs. For instance, a number of antioxidants may not work properly, simply because the cell has its own ability to regulate their redox status and the level of antioxidants and, thus, does not respond to treatment as expected. However, there are some molecules that are not only antioxidants but also energy molecules, and which may prove more effective. For example, pyruvate has long been known to be secreted extracellularly and protect cells from oxidative stress (402) by reacting directly with oxygen radicals such as hydrogen peroxide and peroxynitrite, thus neutralizing their toxic action and enhancing the endogenous glutathione system (518). Its more stable derivates sodium or ethyl pyruvate have beneficial effects in the models of neurodegeneration by also acting as an anti-inflammatory drug (183, 339, 385, 475). Therefore, pyruvate may represent a multi-functional molecule targeting different aspects of ALS. Similar considerations have triggered attempts with lithium carbonate, as at low doses, chronic lithium administration seems to reduce aggregates of misfolded proteins through the induction of autophagy. Furthermore, lithium has been shown to have neuroprotective effects in several models of neurodegeneration through a variety of mechanisms, including the rescue of deficits in axonal transport, GSK3b inhibition, modification of N-methyl-D-aspartate-subtype glutamate receptor function, activation of cell survival factors, induction of neurotrophic proteins, up-regulation of heat-shock protein and Bcl-2, and enhancement of neurogenesis [(8) and refs therein]. However, after a first report on the positive effects in mice and in a small group of patients (176), this treatment failed in three different clinical trials (5, 92, 371), ruling out that this failure may be explained simply by poor design of the trial, and pointing to the difficult reproducibility of positive results even in mice (196, 423). Nonetheless, due to the appealing rationale of this treatment, a fourth trial is ongoing (8).

It should be noted that drugs recently used in clinical trials, that is, olesoxime (Mitotarget) and Dexpramipexol (Biogen-Idec), primarily aimed at stabilizing mitochondrial function (82). Olesoxime did not demonstrate a significant increase in survival *versus* placebo (www.trophos.com), while an encouraging effect of dexpramipexole in subjects with ALS was observed, strongly supporting further testing of this drug in ALS (113). These trials have been designed to include riluzole-treated and -untreated patients, because interactions with the standard drug is likely to occur; any scientific endeavor that develops ALS therapeutics should, therefore, include *in vitro* and *in vivo* animal and human test series with and without riluzole in a clinically relevant concentration.

### B. New strategies

1. Growth factor therapies in ALS. It has long been known that growth factors have pleiotropic actions, ranging from survival effects to the stimulation of neurite outgrowth and axon guidance, synapse formation and maintenance, as well as proliferation, migration, and differentiation of precursor cells (231). For all these reasons, and since it is suspected that a lack of neurotrophic support might contribute to the pathogenesis of ALS, the therapeutic potential of growth factor administration has been the subject of intensive research for the treatment of this disease. Of all the factors tested, many gave promising results in preclinical trials, although the transfer of these results to patients has been proved to be difficult and less effective. Three growth factors better illustrate this point: brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and IGF-1. BDNF and CNTF have been early characterized for their ability to sustain the survival of MNs in cell culture or *in vivo* (197, 470, 471). However, clinical trials testing their therapeutic potential have failed to give any benefit, and also evidenced some adverse effects (2, 34, 274). A similar outcome was observed for IGF-1. Indeed, the delivery of IGF-1 in mouse models of SOD1-linked ALS showed protective effects, with an improvement of pathological phenotypes (139, 281, 390), thus arguing for a benefit to come from the treatment of patients.

Unfortunately, subcutaneously delivered IGF-1 was not effective in patients in a 2-year ALS trial (487).

The reasons for the apparent inefficacy of growth factor treatment in patients might be very complex, and include the limitations of the preclinical models used so far (71), and some weakness in the setting up of the clinical trials themselves. However, the most obvious problem deals with the protocols of administration, which fail to provide a constant rate of growth factor availability during the whole course of the disease. For this reason, a major effort, during the last years, was dedicated to developing gene therapy with viral vectors and cell transplantation. With these new methodologies, it will be interesting to test the potential clinical benefit that many other growth factors, such as glial cell-derived neurotrophic factor (GDNF), VEGF, GCSF, hepatocyte growth factor, fibroblast growth factor, and others [accurately reviewed in (231)], have shown to exert on preclinical models of the disease.

2. Gene therapies in ALS. With regard to other neurodegenerative diseases, gene therapy represents a valuable option for ALS, as many neuro-protective factors as well as neuro-toxic factors that likely play a major role in the disease have been identified so far, and are ideal candidates for a gene therapy approach. Moreover, since the recognition of the multi-systemic origin of ALS, the spectrum of targetable cells is enormously increased, offering new possibilities to therapies. These things said, the achievements of any success in gene therapy is strictly dependent on the way and level target tissues are reached, and given the particularity of the network of tissues in the major affected tissue in ALS, that is, the cortex, brainstem, and spinal cord, an efficient protocol of gene delivery to the spinal cord MNs and/or glia still needs to be developed. Up to date, different kinds of viral vectors developed for their use in the CNS have been utilized with encouraging results. Intra-muscular or intra-neural administration of adeno-associated viral vectors (AAV), whose properties have made this vector system an excellent choice for CNS gene therapy (358), has produced significant results in mice (281) and shown its potentiality also in monkeys (530). An intra-spinal injection, which offers the advantage of directly targeting the affected tissue, has also been tested (24, 177, 326), and a Phase 1 trial for intra-spinal cellular delivery in ALS patients is ongoing (157). As an alternative to overcome the limitations of an in-site delivery of such therapeutic tools, which tend to remain confined in the proximity of the injection site, AAV9 have started being tested. Indeed, once injected intrathecally or intravenously, these AAVs are able to cross the blood-brain barrier, to spread from the site of administration, and to transduce MNs (147, 207, 483) even in large animals (158), thus promising the high translational potential of these tools to diseases that need diffuse gene delivery, such as MNDs.

As discussed in the previous paragraphs, although other and even opposite mechanisms could be evoked, a toxic gain of function is likely responsible for SOD1-linked ALS, and possibly involved in TDP-43 and FUS/TLS-linked fALS. Decreasing the levels of these proteins, by RNA interference (RNAi)-based down-regulation of the involved genes, therefore, seems an obvious approach for improving the ALS condition or, at least, for slowing its progression. Early reports showed the feasibility of such an approach in SOD1 mice, as disease phenotypes in these animals are ameliorated, even at a high extent, by decreasing the levels of expressed SOD1 by lentiviral vector-mediated RNAi (437, 438). On this basis, a clinical trial using antisense oligonucleotides that decrease SOD1 is ongoing (157). However, recent data from SOD1 mice transduced with AAV6-shRNA against SOD1 could not reproduce the therapeutic benefits of the former studies, thus once again stressing the complexity of gene delivery for gene targeting, in general, and SOD1 silencing, in particular (529, 531).

3. Stem-cell therapies in ALS. The replacement of dead MNs, or even the protection of surviving MNs from death, is a major challenge for stem-cell-based therapies in the treatment of ALS. Both the approaches are based on the possibility to generate differentiated functional MNs to refill the population of lost MNs, or various other types of non-neuronal cells that are able to act as nurse cells and provide support to the surrounding MNs (206). Recent advances in stem-cell research have provided a significant amount of various undifferentiated or poorly differentiated precursor cells that potentially fulfill these needs and which, only in some cases, have proved to be effective in ALS preclinical tests. As described in the next paragraph, it is clear that overall, past experience evidentiated the extreme difficulty of a replacement approach, while a cell-based support therapy seems much more promising on the basis of results in experimental models.

Embryonic stem cells (ESCs) have shown to acquire MN phenotypes, including the ability to form functional motor units, when differentiated *in vitro* (580). Moreover, when transplanted *in vivo*, they have improved the motor behavior of the G93A-SOD1 rat model of ALS (341). However, in this experimental setting, *de novo* neurite outgrowth from grafted cells to muscles was not evident, thus suggesting that the protective effect originates from an immunomodulatory and/or trophic action of grafted cells. Similarly, neural stem cells (NSC), that is, stem cells of a neuronal origin that are able to give rise to neurons, astrocytes, and oligodendrocytes (519), have been transplanted in G93A-SOD1 ALS mice, giving positive outcomes in the disease course (101). Interestingly, an analysis of the spinal cord of treated animals revealed a significant proportion of donor cells differentiated in neurons and also MN-like cells. In the same animals, moreover, VEGF and IGF1 were significantly modulated. Thus, both neurogenesis and growth factor release can be achieved by NSC transplantation.

Trophic support from non-neuronal cells has been experimented with in ALS models using different types of stem cells. Among them, the cells of a myeloid origin, which are microglia precursors, have been tested. The transplantation of bone marrow (BM) cells in PU.1 knock-out ALS mice extends their survival, and similar conclusions were obtained in other studies employing BM-derived cells (100, 102, 406).

Mesenchymal stem cells (MSC), non-hematopoietic stem cells isolated from the BM, have also been used for their ability to target areas of damage and to exert a trophic action in their vicinity. Indeed, MSCs transplanted in animal models of ALS have provided some beneficial effect (55, 293, 605). Glial precursor cells transplantation in G93A-SOD1 rats extended the survival and disease duration, attenuated MN loss, and slowed declines in motor function (327). In this experimental setting, transplanted cells efficiently differentiated into astrocytes and reduced microgliosis in the cervical spinal cords,

thus fostering the efficacy of transplantation-based astrocyte replacement.

*Ex vivo* gene therapy is another promising approach for the delivery of growth factors to sites of damage. Human neural precursor cells or human MSCs engineered to secrete GDNF and transplanted, respectively, in the spinal cord and muscles, exert protecting effects on dying MNs and improve MN survival and function in a rat model of ALS (511, 512). Transplantation in the spinal cord of G93A-SOD1 mice of human NSCs engineered to express BDNF, IGF-1, VEGF, neurotrophin-3 (NT-3), or GDNF decreases MN loss, although a significant improvement in motor performance or in lifespan is not observed (416). However, the intrathecal transplantation of human NSCs overexpressing VEGF provides behavioral improvement, disease onset delay, and survival extension in transgenic ALS mice, indicating a better outcome of this modality of treatment (245). Further, direct muscle delivery of GDNF with engineered human MSCs improves MN survival and function in a rat model of fALS (511).

New and exciting studies have shown the possibility to reprogram differentiated adult cells into pluripotent cells, which can be, thus, re-directed along various differentiation lineages. This was achieved in a seminal paper by Takahashi and Yamanaka (514) by forced expression in fibroblasts of the transcription factors Klf-4, Sox-2, Oct-4, and Myc, and was lately confirmed in other article using a different combination of transcription factors (351). These iPSCs present many advantages over traditional stem cells. Indeed, the possibility to collect cells from an individual and generate differentiated cells overcomes the major problems of other stem-cell-based therapies, including the risk of rejection as well as ethical issues.

Dimos *et al.* have shown that human fibroblasts which were harvested from an ALS patient can be differentiated into MNs (137). In a similar approach, gene delivery of MN-specifying transcription factors to human ESCs results in rapid and efficient generation of MNs (235).

Finally, MNs derived fron iPSCs have been used for basic studies conducted on the molecular mechanism involved in ALS and to characterize new compounds for the treatment of this disease (375). Overall, these observations strongly propose the use of iPSCs methodology for disease modeling platforms or as a potential source for cell-based therapies.

## VII. Concluding Remarks

The discovery of new genes associated with the familiar form of the disease has reinforced the concept of ALS as a complex disease, in a previously unsuspected continuum with FTD, in which at least three major cellular pathways play a determinant role: oxidative stress and mitochondria dysfunction, ER stress and defects causing protein aggregation and accumulation, and RNA processing. The first two pathways are clearly linked in more than one way (Fig. 9), and, therefore, it seems reasonable to assume that whatever is the pathogenic cause determining the entrance in the oxidative stress-mitochondria-ER-UPR pathway, several neurotoxic events may follow (*e.g.*, calcium disturbance, axonal transport defects) that will inevitably lead to the activation of non-cell autonomous MN dysfunction.

The relevance of the third pathway in ALS is suggested by several observations. Not only ALS mutations affect TDP-43 and FUS/TLS, which are clearly two proteins involved in RNA metabolism, but also affect other genes (SETX, ANG, and C9ORF72) that may be involved in RNA metabolism as well. Furthermore, patients with SMA, another genetic MN-specific disease, carry a defect in SMN, which is another protein involved in RNA splicing, thus making it tempting to speculate that MNs are particularly sensitive to defects in RNA metabolism.

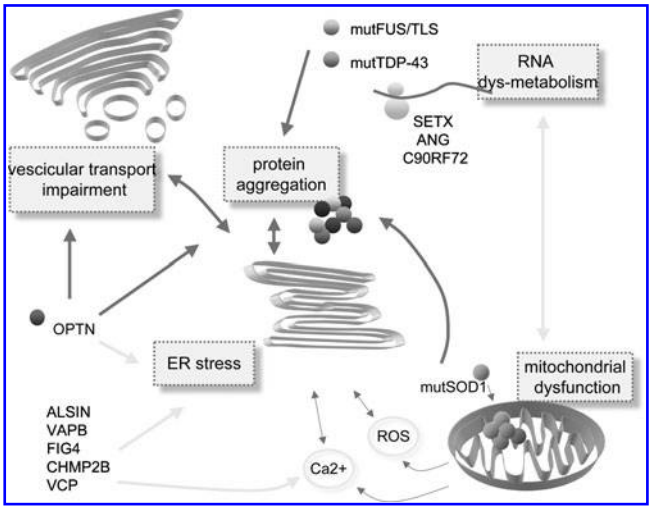


**FIG. 9. A possible network of major cell pathways affected in ALS.**

The third pathway, however, is hardly in an obvious network with the first two, apart from the fact that RNA molecules also need to be transported, for instance, to the pre synaptic end of NMJs.

A possibility to be validated is suggested by our recent work, demonstrating that in two models of oxidative stress and mitochondrial damage (treatment with Complex I inhibitor paraquat and expression of mutant SOD1), dysregulated expression and alternative splicing of a selected population of genes takes place (325). These observations disclose a new, previously unappreciated role for mitochondrial damage in ALS and possibly offer a unifying view of the pathogenesis of ALS. Whether this may help in finding new approaches for the treatment of this disease remains to be seen.

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**Abbreviations Used**

[PI(3,5)P2)] = phosphatidylinositol 3,5-bisphosphate
AAV = adeno-associated viral vectors
ALS = amyotrophic lateral sclerosis
AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propinate
ANG = angiogenin
ATF6 = activating transcription factor 6
ATXN2 = ataxin 2
BDNF = brain-derived neurotrophic factor
BM = bone marrow
C/EBP = CCAAT/enhancer binding protein
CaMKII = Ca-calmodulin-dependent kinase II
CCS = copper chaperone for superoxide dismutase
CHMP2B = charged multivesicular protein 2B
CHOP = C/EBP homologous protein
CLIP = crosslinking and immunoprecipitation
CMT4J = Charcot-Marie-Tooth disease type-4J
CNS = central nervous system
CNTF = ciliary neurotrophic factor
CSF = cerebral spinal fluid
DAO = D-amino acids oxidase
EAAT = excitatory amino acid transporter
ER = endoplasmic reticulum
ELP3 = elongator protein 3 homolog
ERMCC = ER-mitochondria calcium cycle
ESC = embryonic stem cells
FAB1 = familial amyotrophic lateral sclerosis (fALS)
FIG4 = phosphatidylinositol 3,5-bisphosphate 5-phosphatase
FTD = frontotemporal dementia
FTLD = frontotemporal lobar degeneration
FUS/TLS = fused in sarcoma/translocated in liposarcoma protein
GCSF = granulocyte colony stimulating factor
GDNF = glial cell-derived neurotrophic factor
GFP = green fluorescent protein
GRD = glycine rich domain
GRR = glycine rich region
Grx2 = glutaredoxin-2
GSH = reduced glutathione
GSSG = oxidised glutathione
GWAS = genome-wide associations studies
HAT = histone acetyl transferases
HDAC = histone deacetylase
HIF = hypoxia-inducible factor
hnRNP = heterogeneous ribonucleoproteins
HspB8 = heat shock protein B8
HuR = Hu protein R
IGF-1 = insulin-like growth factor 1
IMS = intramembrane space
iPSCs = induced pluripotent stem cells
IRE = iron regulatory element
ITPR2 = 1,4,5-triphosphate receptor 2 gene
JNK = C-Jun N-terminal kinase
KIFAP3 = kinesin associated protein 3
LAMP-2 = lysosomal-associated membrane protein 2
LC3 = microtubule-associated protein1 light chain 3
LCCS1 = lethal congenital contracture syndrome 1
miRNA = microRNA
MN = motor neuron
mPRP = mouse prion promoter

**Abbreviations Used (Cont.)**

MRI = magnetic resonance imaging
MSC = mesenchymal stem cells
MTS = mitochondrial targeting sequence
nAChR = neuronal nicotinic acetylcholine receptor
NES = nuclear export signal
NF-$\kappa$B = nuclear factor kappa B
NFL = low molecular weight neurofilament
NIPA1 = nonimprinted in Prader-Willi/Angelman syndrome 1
NLS = nuclear localization signal
NMJ = neuromuscular junction
NT-3 = neurotrophin-3
Ocln = occludin
OPTN = optineurin
PERK = protein kinase like ER kinase
PGRN = progranulin
PINK1 = PTEN-induced kinase
PLS = primary lateral sclerosis
POAG = primary open-angle glaucoma
rER = rough ER
RNAi = RNA interference
ROS = reactive oxygen species
RRM = RNA recognition motif
sALS = sporadic amyotrophic lateral sclerosis
SCA2 = spinocerebellar ataxia type 2
SETX = senataxin
SG = stress granule
shRNA = short hairpin RNA
Sig-1R = Sigma-1 receptor
SIRT = sirtuin
SMA = spinal muscular atrophy
SMN = survival of motor neuron
SOD1 = Cu, Zn superoxide dismutase
SPG11 = spatacsin
TAR = trans active response
TDP43 = transactive response DNA-binding protein
Thy1 = thymocyte differentiation antigen 1 promoter
tTA = inducible expression by tetracycline transactivator
UBQLN2 = ubiquilin 2
UPR = unfolded protein response
VAC14 = voltage-dependent anion channel (VDAC)
VAPB = vesicle-associated membrane protein (VAMP)-associated protein (VAP) B
VCP = valosin-containing protein
VEGF = vascular endothelial growth factor
VPA = valproic acid
XBP-1 = x-box binding protein
ZO-1 = zona occludens