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Mitochondrial Involvement in Amyotrophic Lateral Sclerosis

Trigger or Target?

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Abstract

Despite numerous reports demonstrating mitochondrial abnormalities associated with amyotrophic lateral sclerosis (ALS), the role of mitochondrial dysfunction in the disease onset and progression remains unknown. The intrinsic mitochondrial apoptotic program is activated in the central nervous system of mouse models of ALS harboring mutant superoxide dismutase 1 protein. This is associated with the release of cytochrome-*c* from the mitochondrial intermembrane space and mitochondrial swelling. However, it is unclear if the observed mitochondrial changes are caused by the decreasing cellular viability or if these changes precede and actually trigger apoptosis. This article discusses the current evidence for mitochondrial involvement in familial and sporadic ALS and concludes that mitochondria is likely to be both a trigger and a target in ALS and that their demise is a critical step in the motor neuron death.

Index Entries: Mitochondria; ALS; apoptosis; SOD1.

Introduction

Amyotrophic lateral sclerosis (ALS) is also known in the United States as Lou Gehrig's disease, named for the New York Yankee baseball player who was diagnosed with the disease in the 1930s. In France, it is known as "Maladie de Charcot," after the French neurologist and neuropathologist Jean-Martin Charcot, who first clearly identified the disease in 1869. ALS is characterized by a progressive selective degeneration and loss of motor neurons in the spinal cord, brain stem, and motor cortex, which lead to muscular weakness, atrophy, spastic paralysis, respiratory failure, and death *(1)*. It is a devastating neurodegenerative disorder, with an incidence of 2 to 3 per 100,000 people per year. The average age of onset is approx 50 yr, but younger cases have also been

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observed. Patients typically succumb within 3 to 5 yr of diagnosis *(2)*.

About 5% of cases of ALS are familial (FALS) and the remainder are sporadic (SALS). With some exceptions, these two forms cannot be distinguished clinically. Approximately 20% of cases of FALS have been associated with more than 100 different mutations mapped to the Cu, Zn-superoxide dismutase *(sod1)* gene on chromosome 21. SOD1 is a 17-kDa protein responsible for the conversion of superoxide radicals to hydrogen peroxide, thus playing a major role in the anti-oxidant defense of the cell *(3,4)*. It has copper and zinc as prosthetic groups, and a specific copper chaperone, termed copper chaperone of SOD1 (CCS), has been identified in yeast *(5)* and mammals *(6)*. Pathogenic SOD1 mutations are predominantly single amino acid replacements that are randomly scattered throughout the structure of this homodimeric 32,000-kDa enzyme. In most families, the inheritance of the SOD1 mutations occurs in an autosomal dominant fashion, although it can be autosomal recessive (http://www.alsod.org).

Gurney and colleagues *(7–9)* produced the first transgenic SOD1 mouse model of ALS. The transgenic SOD1 G93A mice express high levels of mutant human SOD1 ubiquitously and develop a progressive motor neuron disease. Conversely, models that express mutant SOD1 exclusively in the motor neurons or in astrocytes neither develop the disease nor manifest motor neuron pathology *(10,11)*. Mutant SOD1 exerts its deleterious effect by a "toxic gain of function" rather than by a loss of anti-oxidant activity *(12)*.

Kong and Xu *(13)* defined four stages of the disease in mice expressing G93A mutant SOD1. The onset of the disease is marked by a relatively rapid decline of muscle strength and an increase in vacuoles in motor neurons derived from degenerating mitochondria, with little motor neuron death. Most motor neurons do not die until the terminal stage, which is approx 9 wk after disease onset. These observations suggest that mutant SOD1 toxicity may be mediated by damage to mitochondria and that this damage could trigger the functional decline of motor neurons and the clinical onset of ALS.

There is currently no effective treatment for ALS, and relatively few clues exist regarding its etiology. Over the past several years, hypotheses regarding the pathogenesis of ALS have focused on oxidative damage *(14)*, protein aggregation *(15)*, neurofilament disorganization *(16)*, mitochondrial dysfunction *(17)*, pro-apoptotic mechanism *(18)*, toxic-modified amino acids produced by cyanobacteria *(19)*, and excitotoxicity *(20)*. This article focuses on the mitochondrial involvement in ALS and critically discusses its role in the disease process.

Ultrastructural Mitochondrial Abnormalities in ALS

Electron microscopy studies have shown abnormal mitochondrial morphology in patients with ALS at early stages of the degeneration of motor neurons *(21)*. Muscle biopsy samples from patients with ALS showed ultrastructural abnormalities of muscle mitochondria, including giant mitochondria, paracrystalline inclusions, and abnormal cristae. These altered mitochondria were observed mainly among subsarcolemmal mitochondrial aggregates *(22)*. These alterations were also found in the transgenic SOD1 mouse model of ALS (G37R and G93A SOD1) where membrane-bound vacuoles in axons, dendrites, and motor neurons appeared to be derived from degenerating mitochondria *(13,23–25)*. The mitochondrial swelling and extensive vacuolization of the motor neuron cytoplasm in mutant SOD1 mice increased with age and accompanied massive motor neuron damage *(24)*. Electron microscopy studies also showed large SOD1-immunoreactive structures associated with vacuolated mitochondrial membranes *(25,26)*. This massive mitochondrial vacuolation resulting from expansion of mitochondrial intermembrane space and extension of the outer membrane was also shown to involve peroxisomes *(27)*.

Abnormal mitochondrial cristae retained cytochrome oxidase activity in the spinal cord of G93A SOD1 mice, although this occurred in a patchy fashion *(28)*. Although they appear early, it is difficult to determine whether mitochondrial structural abnormalities preceded other cellular changes, such as cytoskeleton alterations or a metabolic crisis, which might not be detected as easily.

Alterations in Respiration and Electron Transport Chain

Mitochondrial electron transport chain activities in muscle biopsies from patients with ALS, animal models of ALS, and cultured cells have yielded differing and controversial results. In one study, oxidative phosphorylation complex I activity was increased in postmortem brain tissue in patients with FALS *(29)*. In another study, complex I and II–III deficiencies were observed in patients with FALS and SOD1 mutations as well as in the transgenic mouse model *(30)*. Impairment of mitochondrial function was observed in skeletal muscle biopsies from patients with ALS *(31–33)*, and altered respiration was described in muscle mitochondria of patients with early stage SALS using the skinned fiber technique *(34)*. Loss of citrate synthase activity (a mitochondrial marker) as well as decreased activities of respiratory chain complexes I and III, II and III, and IV suggested a loss of mitochondria in spinal cords of patients with ALS *(35)*. Mitochondrial respiration, electron transport chain, and adenosine triphosphate synthesis were defective in G93A mice at the onset of the disease *(17)*. In the same animal model, mitochondrial electron transport chain activities were decreased in the spinal cord ventral horn prior to disease onset and during disease progression *(36)*. These alterations in mitochondrial function in G93A SOD1 mice appeared to be specific and preferentially targeted to the central nervous system (CNS; ref. *28*).

Complexes II and IV activity were decreased in a cell culture model of FALS *(37)*. Cytoplasmic hybrids with mitochondrial DNA (mtDNA) from patients with ALS exhibited altered mitochondrial ultrastructure and biochemical function *(38)*, but these findings were not reproduced by other workers using a different nuclear background *(39)*. Diminished levels of mtDNA were observed in skeletal muscle of patients with SALS *(33)*. Other reports have suggested that a 4977-basepair deletion in mtDNA may be associated with the occurrence of SALS *(40,41)*. However, no clear relationship has been demonstrated between neuronal cell death in ALS and accumulation of mtDNA deletions or mtDNA genome alterations *(42,43)*. The lack of maternal transmission also contradicts the concept that mtDNA haplotypes are major susceptibility factors in ALS *(44,45)*.

Motor neurons require a high supply of energy and would be sensitive to any disturbance in mitochondrial energy production—perhaps more sensitive than other neuronal groups *(42)*. Work from our laboratory showed that only one segment of the respiratory chain was altered in the CNS of the G93A SOD1 mice; this segment involved cytochrome-*c* and cytochrome-*c* oxidase. A similar abnormality was observed in the wobbler mice, another model of motor neuron degeneration *(46)*. Despite this early defect in complex IV respiration, we did not observe a defect in cytochome oxidase enzyme activity until the age at which animals exhibited overt symptoms *(28)*. Therefore, our results suggest that a defect in the specific activity of cytochrome oxidase is a secondary effect of the observed mitochondrial degeneration. As discussed later, the early respiratory defect was associated with an impairment of cytochrome-*c* association with the inner mitochondrial membrane (IMM) *(28)*.

The fact that defects in oxidative phosphorylation (OXPHOS) function are observed in brain and spinal cord homogenates in transgenic mice models suggests that these defects may involve other CNS cell types in addition to motor neurons. However, the data regarding

defects in oxidative phosphorylation do not exclude the suggestion that the biochemical defects are secondary to the neurodegenerative process in both neural and muscular tissues.

Reaction Oxygen Species Production and Lipid Peroxidation in Amyotrophic Lateral Sclerosis

Mitochondrial respiration is the main source of reactive oxygen species (ROS) in the cell, and ROS levels tend to increase when respiration is impaired *(47)*. Both the involvement of mitochondria and the association of SOD1 with ALS suggest that oxidation of macromolecules could have a role in the pathogenesis of ALS. In patients with SALS, both lipid and protein oxidation are enhanced in the spinal cord motor neurons and glial cells, suggesting that the formation of these products is implicated in motor neuron degeneration *(48)*. Markers of oxidative stress and immune activation were significantly elevated in postmortem ALS tissue in the CNS *(49)*, and abnormally increased blood levels of ROS and lactate production may indicate a close relationship between mitochondrial function and oxidative stress in ALS *(50)*. These increases in ROS and products of oxidation have been observed both in postmortem samples and in experimental models for ALS and may result from an altered metabolism of copper and iron ions *(51)*. Although some reports suggest that the copper at the active site of SOD1 is not involved in the pathogenesis *(52)*, copper, which is also allosterically bound to SOD1, may still participate in peroxidative reactions *(53)*.

ROS and oxidation of protein, DNA, and membrane phospholipids were significantly elevated in mutant SOD1 transgenic mice *(54)*. Staining with an antibody for nitrotyrosine, a marker of oxidative stress, showed a large increase in the same mouse model *(55)*. Furthermore, an increase in spinal cord lipid peroxidation was found in the FALS transgenic mouse model, preceding the onset of ultrastructural or clinical motor neuron disease, with the greatest intensity of motor neuronal lipid peroxidative injury occurring at the active phase of disease progression *(56)*. Additionally, lipid peroxidation—particularly of cardiolipin—has been reported in mitochondria of G93A mice as evidence of oxidative damage to mitochondrial lipids *(17, 28,57)*.

Additionally, alterations in ROS scavenging enzymes, including peroxiredoxin 2 (Prx2, thioredoxin peroxidase) and glutathione peroxidase1 (GPx1), may also participate in ALS. Lewy body-like hyaline inclusions in the spinal cords of patients with FALS as well as in transgenic rats expressing human SOD1 with H46R and G93A mutations showed co-aggregation of Prx2/GPx1 with SOD1. These may lead to the breakdown of the redox system itself, suggesting that SOD1 aggregates may indirectly affect the cellular ROS scavenging system *(14)*. Locally increased SOD2 immunoreactivity of astrocytes in FALS and SALS *(58)*, motor neurons *(59)*, and a patient's cell lines *(60)* has been demonstrated, but the results in CNS cells *(61)* and in skeletal muscle *(33)* were controversial.

These findings suggest a relationship between enhanced oxidative stress and mutant SOD1-mediated motor neuron degeneration. ROS-induced peroxidative damage of mitochondria has been also proposed to contribute to aging, ischemia/reperfusion, and chronic degenerative disease *(62)*.

Mitochondrial Localization of Superoxide Dismutase 1

SOD1 is localized primarily in the cytosol of eukaryotic cells *(63)* and was also found in nucleus and peroxisomes *(64)* as well as in bacteria *(65,66)*. Additionally, many studies have demonstrated its presence in rat liver mitochondria *(67,68,69)* and, possibly, in liver lysosomes *(70)*. A fraction of endogenous mouse SOD1, transgenic wild-type SOD1, and mutant human G93A SOD1 colocalize with mitochondria in spinal cord at similar concentrations as

in the cytoplasm (as detected by immunofluorescence confocal microscopy and immunoelectron microscopy studies; ref. *71*) and also was found to localize to mitochondria and peroxisomes in rat brain, liver, and in human fibroblasts *(64)*.

Initial studies showed that SOD1 was confined to the intermembrane space of yeast mitochondria *(72)* and of liver and brain mitochondria from G93A SOD1 and wild-type SOD1 transgenic mice *(17)*. These findings were supported by the fact that SOD1 was found to colocalize with cytochrome-*c* and not with SOD2 *(26)*. However, a recent report demonstrated that both wild-type and mutant human SOD1 displayed a complex intramitochondrial compartmentalization in brain, localizing not only in the mitochondrial outer membrane and in the intermembrane space but also in the mitochondrial matrix *(73)*. Such matrix localization was not found in rat liver mitochondria *(69)*. Another group of reports has suggested that SOD1 is present only at the mitochondrial outer membrane *(74,75)*. In any case, SOD1 appears to be more concentrated in brain mitochondria than liver mitochondria. The fact that mutant SOD1 is increased in mitochondria of neural tissue provides some tantalizing clues to the neurodegenerative process in FALS *(73)*.

Some reports have found only mutant SOD1, but not wild-type SOD1, in spinal cord mitochondria of transgenic mice *(74)*, whereas others have found both wild-type and mutant SOD1 localized in mitochondria of spinal cord and in other CNS regions *(17,26,28,69,71,73)*. The mitochondrial localization of mutant human SOD1 suggests that mitochondria plays an important role in neurodegeneration and may be critical in triggering motor neuron death in FALS.

Superoxide Dismutase 1 Aggregation Outside and Inside of Mitochondria

The formation of aggregates of human SOD1 is believed to be a major toxic factor leading to neuronal cell death *(76)*. The aggregation is likely to result from protein misfolding and failure of the ubiquitin-proteasome proteolytic pathway to dispose of excessive abnormal protein *(77)*. Ubiquitin-immunoreactive intraneuronal inclusions have been described in ALS and may represent accumulations of altered or abnormal neuronal proteins resistant to degradation *(78)*.

Aggregation of SOD1 may significantly contribute to the death of motor neurons expressing mutations associated with FALS, and the mechanisms leading to aggregation may pertain to the specific vulnerability of motor neurons in this disease *(79)*. Different studies have demonstrated the presence of cytosolic filamentous aggregates in the spinal cord of G93A mice in the late phase of the disease *(80)*. These SOD1-containing aggregates were associated with different site mutations *(76)*. Misfolded mutant SOD1 and aggregates were found in high levels in the CNS *(81)*.

Interestingly, intracytoplasmic mutant SOD1 aggregates were suggested to be a product processed by a retrograde transport of microtubules forming an aggregosome *(82)*, and the presence of aggregates containing electron-dense cores was frequently observed in torpedo-like swollen axons consisting of accumulated neurofilaments, potentially causing impairment of axonal transport by physical blockage *(83)*.

Abnormal cytoplasmic aggregation of mutant SOD1 has also been observed in cultured spinal motor neurons, dorsal root ganglion, and hippocampus neurons *(79)*. Mutant SOD1 aggregates were found in association with the endoplasmic reticulum after inducing endoplasmic reticulum stress in complementary DNA-transfected COS7 cells. These cells showed an aberrant intracellular localization of mitochondria and microtubules, which might lead to a functional disturbance of the cells *(84)*.

Mutant SOD1 in the spinal cord of both humans and mice models of FALS was hypothesized to aggregate preferentially on the cytoplasmic face of the outer mitochondrial

membrane, possibly in association with mitochondrial import pores *(74)*. Macromolecular aggregates of mutant SOD1 were found in brain mitochondria matrix in ALS mice *(73)*. A recent report *(75)* showed that the anti-apoptotic protein Bcl-2 interacts with both wild-type and mutant SOD1. However, this association was only observed with the mutant SOD1, both in vitro and in spinal cord of transgenic SOD1 mice. These mutant SOD1/Bcl-2-containing aggregates were present in mitochondria from spinal cord but not liver.

Heat shock proteins (HSPs) function as molecular chaperones that recognize and renature misfolded proteins and maintain proteins in an appropriate conformation *(85)*. HSPs exhibit cytoprotective functions in stress tolerance *(86)*. Increasing the levels of the stress-inducible cytosolic chaperone 70-kDa HSP (HSP70) by gene transfer markedly reduced the formation of mutant SOD1-containing proteinaceous aggregates in cultured primary motor neurons that expressed G93A SOD1 and prolonged their survival *(87)*. Furthermore, combined effects of HSP70 and its cofactor, HSP40, reduced intracytoplasmic aggregates, improved neurite outgrowth, and prevented cell death *(88)*. HSP25, which is constitutively expressed in motor neurons, is decreased in G93A SOD1 mice prior to the onset of motor neuron death and muscle weakness and before the appearance of SOD1 aggregates *(89)*. More recently, a significant decrease in chaperone activity in cell lysates from spinal cord of ALS mice (before the onset of muscle weakness and significant motor neuron loss) was described and persisted throughout the late stages. However, this decrease was observed in cytosolic, but not in the mitochondrial or nuclear fractions *(90)*. This does not rule out the possibility that dysfunction of cytosolic chaperones causes mitochondrial dysfunction and degeneration, because mitochondrial protein importation depends on the functions of cytosolic chaperones *(91)*. Moreover, protein aggregates at the mitochondrial surface may directly affect mitochondrial function. It also appears that the HSPs–SOD1 complex recruits other proteins present in the neuroblastoma cell and, presumably, in motor neurons to form sedimentable aggregates *(92)*.

Apoptotic Mechanisms in Amyotrophic Lateral Sclerosis

Both cell culture systems and mouse models have provided strong evidence that motor neuron death in ALS involves the intrinsic apoptotic cascade. Cultured spinal motor neurons, dorsal root ganglion, and hippocampus neurons transfected with the ALS SOD1 mutations were susceptible to toxicity and apoptotic cell death *(79)*. One cell culture model of FALS used a motor-neuron-like cell line (NSC34) that expressed normal or mutant human SOD1 at levels similar to those observed in the human disease. In this model, cells expressing mutant SOD1 showed increased cell death when exposed to oxidative stress by serum withdrawal or when the glycolytic pathway was pharmacologically inhibited, whereas the presence of normal human SOD1 exerted a protective effect *(37)*. When the mutant SOD1 was targeted to mitochondria in a neuronal cell line, a release of mitochondrial cytochrome-*c* was observed, followed by the activation of the caspase cascade and induction of neuronal cell death without cytoplasmic mutant SOD1 aggregate formation. Nuclear and endoplasmic reticulum localization of mutant SOD1 did not induce cell death *(93)*.

The evidence for apoptosis involvement in mouse models is also strong. The pro-apoptotic protein Bax translocates from the cytosol to the mitochondria, whereas cytochrome-*c* translocates from the mitochondria to the cytosol in spinal cords of transgenic SOD1 mice in parallel with the neurodegenerative process. Caspase-9, followed by caspase-7, is activated in the spinal cord of transgenic mutant SOD1 mice *(94)*. An early cleavage of caspase-12, which resides in the endoplasmic reticulum, is also observed in the spinal cord during the course of the disease. In addition to

caspase-12, caspase-9 and caspase-3 were also activated in the transgenic ALS mice *(55).*

Co-immunoprecipitation experiments have demonstrated that Bax–Bax interactions are greater in the mitochondria-enriched membrane compartment of ALS motor cortex compared to controls, whereas Bax–Bcl-2 interactions are reduced in the membrane compartment of ALS motor cortex, suggesting an increased susceptibility of ALS mice to apoptosis. The anti-apoptotic protein Bcl-2 is also decreased in the mitochondria-enriched membrane compartment of vulnerable regions in human ALS spinal cord anterior horn and motor cortex but is increased in the cytosol. Bcl-xL levels are unchanged in both subcellular compartments *(95).*

A study published by Vukosavic and colleagues *(96)* demonstrated significant alterations in the expression of key members of the Bcl-2 family associated with mutant SOD1 deleterious effects. They showed that in presymptomatic transgenic mutant SOD1 mice, expression of Bcl-2, Bcl-xL, Bad, and Bax did not differ from that in nontransgenic mice. However, symptomatic mice showed reduced expression of Bcl-2 and Bcl-xL. Overexpression of Bcl-2 increases the formation of Bcl-2:Bax heterodimers, thus abolishing the Bax pro-apoptotic property. Concurrently, overexpression of the anti-apoptotic protein Bcl-2 attenuates neurodegeneration and delays activation of the caspases in the G93A SOD1 mice *(97).*

More recently, Pasinelli et al. *(75)* demonstrated that although the wild-type SOD1 is anti-apoptotic, mutant SOD1 promotes apoptosis and that both bind the anti-apoptotic protein Bcl-2 in mouse and human spinal cord, providing more evidence of a direct link between SOD1 and an apoptotic pathway. More support for these findings came from a study by Inoue et al. *(98)* that suggests that caspase-9 plays a crucial role in disease progression in ALS, showing that it is activated in spinal motor neurons of human ALS subjects. Moreover, by inhibiting caspase-9 activation, the progression of the disease is attenuated in spinal motor neurons of mutant SOD1 mice.

Therefore, it appears that a programmed cell-death mechanism involving cytosol-to-membrane and membrane-to-cytosol redistribution of cell-death proteins and caspase activation participates in the pathogenesis of ALS and that the mitochondria-dependent apoptotic pathway contributes to the demise of motor neurons in ALS. However, because mitochondrial abnormalities are part of the apoptotic process, it is difficult to determine whether the numerous observations of mitochondrial abnormalities result from an active apoptotic pathway. Our recent observations of very early dissociation of cytochrome-*c* from the IMM in the CNS of the G93A SOD1 mice suggest that the gross structural and biochemical mitochondrial abnormalities could result from the apoptotic program.

The Cytochrome-*c* Involvement in Amyotrophic Lateral Sclerosis

Kirkinezos et al. *(28)* found that electron transfer at the level of OXPHOS complex IV was impaired in G93A SOD1 mice as early as age 30 d, well before the onset of any signs of neuronal degeneration. By using reduced cytochrome-*c* as an electron donor, they were able to bypass the complex IV respiration defect in brain mitochondria. Furthermore, they showed that loss of cytochrome-*c* from brain mitochondria in mutant SOD1 transgenic mice was preceded by a decreased association of cytochrome-*c* with the IMM *(28).* Interestingly, cytochrome-*c* is located in the same compartment as a portion of SOD1. It is known that chemical modifications, such as peroxinitration, reduce cytochrome-*c* function, inhibit respiration, and can stimulate apoptosis *(99)* and that cytochrome-*c* association with the IMM depends on the integrity of the membrane lipids—particularly on intact cardiolipin, an IMM lipid *(100–102).* Mitochondria-mediated ROS generation can affect the activity of OXPHOS enzymes via

peroxidation of cardiolipin, which is needed for the optimal functioning of some enzyme complexes *(62)*. Cardiolipin is altered during neuronal apoptosis in a process mediated by ROS *(103)*, and the decrease in the level of unmodified cardiolipin affects cytochrome-*c* binding to the IMM, leading to higher levels of soluble cytochrome-*c* in the mitochondrial intermembrane space and the induction of apoptosis *(104)*. Furthermore, cardiolipin peroxidation might have an initiating role in the liberation of cytochrome-*c* from the IMM and in the opening of the permeability transition pore via inactivation of the adenine nucleotide translocator *(105)*.

The Role of Mitochondrial Calcium in Amyotrophic Lateral Sclerosis

Intracellular Ca^{2+} levels have long been recognized as triggers of cell death and as modulators of mitochondria-mediated apoptosis *(104)*. Perturbed cellular calcium homeostasis has been implicated in both apoptosis and necrosis, and the role of altered mitochondrial calcium handling in the process of cell death is under active investigation *(20)*.

The content of glutamate, which mediates increases in intracellular Ca^{2+} in motor neurons, was found to be either decreased in several regions of the CNS *(106,107)* or increased in the cerebrospinal fluid of patients with ALS *(108)*. Approximately 60 to 70% of patients with SALS have between 30 and 95% loss of the astroglial glutamate transporter excitatory amino acid transporter 2 (EAAT2; also called glutamate transporter 1) in motor cortex and spinal cord, and similar changes have been observed in the mutant mouse model of ALS *(109–112)*. The loss of EAAT2 may result from aberrant messenger RNA, possibly caused by RNA processing errors *(113)*, but not from abnormal splice variants *(114, 115)*. Abnormal splice variants could increase extracellular glutamate in patients with ALS, leading to excitotoxic neuronal degeneration *(116)*. Focal loss of EAAT2 in the ventral horn of the spinal cord of rats that overexpressed mutant SOD1 appeared before motor neuron/axon degeneration and were increased at the end-stage of the disease, suggesting a role for this protein in the events leading to cell death in ALS *(117)*.

In the mouse model of ALS, motor neurons exhibited greatly increased vulnerability to glutamate toxicity mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-type glutamate receptors, and this was associated with enhanced ROS production, sustained elevations of intracellular calcium levels, and mitochondrial dysfunction. Pretreatment of cultures with vitamin E, nitric-oxide-suppressing agents, peroxynitrite scavengers, and estrogen protected motor neurons against excitotoxicity *(118)*. AMPA receptors are major mediators for fast excitatory neurotransmission in the mammalian CNS and are composed of a heteromeric complex of four subunits (GluR1–GluR4); the absence of GluR2 renders the receptor Ca^{2+}-permeable *(119)*. A defect in the editing of the messenger RNA encoding the GluR2 subunit of glutamate AMPA receptors in the spinal motor neurons of individuals affected by ALS interferes with the correct functioning of the glutamate receptors and may be a contributory cause of neuronal death in patients with ALS *(120)*. This suggests that Ca^{2+}-permeable AMPA-receptor-mediated excitotoxicity is closely linked to the vulnerability of spinal motor neuron in ALS. Additionally, a recent report evaluated the contribution of overexpression of motor neuron Ca^{2+}-permeable GluR2 AMPA-type glutamate receptors to SOD1-related motor neuronal death by generating a mouse model with a cholinergic neuron-specific promoter. These mice showed spinal motor neuron receptors with significantly reduced Ca^{2+}-permeability, and crossing the G93A SOD1 transgenic mouse model of ALS with cholinergic neuron-specific promoter-GluR2 mice led to marked delay of disease onset and mortality *(121)*.

Motor neurons lack important Ca^{2+}-buffering proteins such as parvalbumin and cal-

bindin D28K, and this may cause the selective vulnerability of motor neurons to the neurodegenerative process by impairing neuronal calcium homeostasis *(122).* In a neuroblastoma cell line with constitutive expression of mutant SOD1, there was a significant loss of mitochondrial membrane potential and a parallel increase in cytosolic Ca^{2+} concentration *(123).* Other studies showed increased levels of Ca^{2+} within the mitochondria in ALS cases *(124).* Mitochondria are the major Ca^{2+} buffer in motor neurons, and defects in mitochondrial function lead to increases in cytosolic Ca^{2+} *(125,126).* Such increases could lead to endoplasmic reticulum stress, activation of Ca^{2+}-signaling cascades, or activation of proteases.

The mitochondrial dysfunction associated with SOD1 mutations may play an important role in disturbing Ca^{2+} homeostasis and increasing ROS production, thereby raising the vulnerability of motor neurons to excitotoxicity. However, we again have a "chicken or egg" paradigm. Does excitotoxicity lead to increase in cytosolic Ca^{2+} and subsequent activation of apoptosis, or does the impaired Ca^{2+}-buffering capacity of mitochondria bring cytoplasmic Ca^{2+} to levels above a pathogenic threshold?

Cytoskeletal Abnormalities and Their Effect on Mitochondrial Function in Motor Neurons

The role of cytoskeletal proteins in ALS remains unclear. Both transgenic mouse models and cell culture studies support the view that peripherin, an intermediate filament protein detected in spheroids, is associated with ALS *(127).* However, a gene knockout approach demonstrated that peripherin was not a key contributor to motor neuron disease caused by mutant FALS SOD1. Alsin, a cytoskeletal protein found to be mutated in some FALS families *(128,129),* caused a progressive motor dysfunction during aging in a mouse alsin knockout model *(130).*

Mouse neuroblastoma Neuro-2a cells transfected with mutated (G37R or G93A) SOD1 exhibited marked retardation in cell growth and G2/M arrest and displayed lower reactivity to phalloidin, indicating that the cytoskeleton was disrupted and that mutated SOD1 was associated with actin *(131).* Furthermore, neurofilament accumulation in mice (the most abundant cytoskeletal structure in large myelinated axons) causes neurodegeneration by disrupting axonal transport, a mechanism that has been associated with neurodegenerative diseases such as ALS *(16,132).* The altered stoichiometry of cytoskeletal protein expression in ALS spinal motors can lead to the formation of neurofilamentous accumulation and aggregates *(133),* and both neurofilaments and anterograde transport are reduced in ventral root axons of transgenic mice that express mutant SOD1 *(134).* Cytoskeleton abnormalities can impair mitochondrial function by restricting axonal transport *(135).* This was demonstrated by a marked accumulation of mitochondria and lysosomes in the proximal axons of patients with ALS *(136).* Mitochondria need to be transported to and from the soma along cytoskeleton tracks, which could be blocked by cytoskeleton disorganization of protein aggregation *(134).*

Notably, mutations in Mitofusin-2, a mitochondrial membrane protein that participates in mitochondrial fusion in mammalian cells *(137, 138),* has been reported to cause another motor neuron disease known as Charcot-Marie-Tooth neuropathy type 2A *(139–141).* Although the mechanisms associated with this defect are unknown, Mitofusin-2 can regulate OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein *(142),* raising the possibility that a bioenergetic defect may cause motor neuron disease.

Treatments Potentially Targeting Mitochondrial Dysfunction

The molecular mechanisms of selective motor neuron degeneration in human ALS remain

largely unknown, and effective therapies are not currently available. However, numerous different therapeutic approaches have been targeted to mitochondrial dysfunction and increased ROS, thereby trying to protect mitochondria against the pro-apoptotic effects of mutant SOD1.

Coenzyme Q10, an essential cofactor of the electron transport chain as well as an important anti-oxidant, was demonstrated to significantly extend survival in a transgenic mouse model of ALS *(143)*. Minocycline mediates neuroprotection in experimental models of neurodegeneration. It inhibits, although not directly, the activity of caspase-1, caspase-3, the inducible form of nitric oxide synthetase, and p38 mitogen-activated protein kinase; when tested in mice with ALS, it delayed disease onset and extended survival. Its action was associated with the inhibition of the mitochondrial permeability-transition-mediated cytochrome-*c* release *(144)*. Caspase inhibition may also have a protective role in ALS, as it was demonstrated in transgenic mice expressing mutant human SOD1. Intracerebroventricular administration of zVAD-fmk, a broad caspase inhibitor, directly inhibited caspase activation and delayed disease onset and mortality *(145)*.

Oral administration of creatine produced a dose-dependent improvement in motor performance and extended survival in G93A SOD1 transgenic mice by protecting mice from loss of both motor neurons and substantia nigra, decreasing the biochemical indices of oxidative damage, and inhibiting the opening of the mitochondrial transition pore *(146)*.

Creatine kinase and its substrates, creatine and phosphocreatine, constitute an intricate cellular energy buffering and transport system of energy production in mitochondria. By inhibiting the opening of the mitochondrial transition pore, creatine administration produced an improvement in motor performance and extended survival in transgenic mice *(146,153)*. The beneficial effect of creatine may be partially mediated by an improvement in the function of the glutamate transporter, which has a high demand for energy and is susceptible to oxidative stress *(154)*. Furthermore, the combination of minocycline and creatine resulted in additive neuroprotection, suggesting it to be a novel potential strategy for the treatment of ALS *(155)*. Conversely, other studies did not show any clinically significant improvement in the function of respiratory muscles in patients suffering from ALS with respiratory distress from the use of creatine *(156)* and did not exert any beneficial effect on muscle function in a transgenic mouse model of ALS *(157)* or in a clinical trial of patients with ALS *(158)*.

Motor-neuron-like cells overexpressing the mitochondrial anti-oxidative genes *MnSOD* and *GPX4* by stable transfection showed significantly increased resistance to mutant SOD1 toxicity. The spin trapping molecule 5′,5′-dimethylpryrroline-*N*-oxide also prevented mutant SOD1-mediated mitochondrial dysfunction and cell death, significantly delaying paralysis and increasing survival *(150)*.

Mitochondrial homeostasis was affected by mutant SOD1-generated ROS; this alteration was reversed by anti-oxidant treatments like *N*-acetylcysteine, which lowers ROS production and returns mitochondrial functional assays to control levels *(147)*. Pramipexole treatment, which was previously shown to reduce oxidative stress and to be neuroprotective in cell and animal models of neurodegeneration, was able to interrupt free radical production in patients with ALS *(148)*.

Treatment with a selective cyclooxygenase-2 inhibitor (Celebrex®) markedly inhibited production of prostaglandin E2 in the spinal cords of ALS mice, thereby delaying the onset of weakness and weight loss and prolonging survival. This effect probably resulted from the inhibition of prostaglandin E2 stimulation of glutamate release from astrocytes and the inhibition of the cyclooxygenase-2-mediated production of pro-inflammatory cytokines, ROS, and free radicals *(149)*. However, a clinical trial of patients with ALS (200 receiving the drug and 100 receiving placebo) failed to show ben-

efit (http://www.alsa.org/patient/drug.cfm?id=47).

Another study demonstrated that vascular endothelial growth factor, a potent stimulator of angiogenesis, delayed progression of symptoms and prolonged survival in a SOD1 transgenic mouse model of ALS, suggesting possible value in the treatment of patients with ALS *(151).* This treatment had an anti-apoptotic effect in the mouse NSC34 motor-neuron-like cell culture system infected with adenovirus containing mutant SOD1, because motor neuron cell death was diminished by pretreating the cells with vascular endothelial growth factor, which induced a dose-dependent resistance to oxidative damage from hydrogen peroxide, tumor necrosis factor-α, and mutant G93A SOD1 *(152).*

HSPs may play an important role in self-defense against intracytoplasmic aggregate formation and may provide a basis for the use of HSPs in developing a treatment for ALS, as it was demonstrated that the combination of HSP70 and HSP40 reduced intracytoplasmic aggregates and markedly improved neurite outgrowth while preventing cell death to a relatively lesser extent *(88).*

Cyclosporin A treatment, which is well-known for its extracerebral effect as an immunosuppressant in organ transplantation, prolonged the survival of ALS transgenic mice when infused into the lateral cerebral ventricle. It is believed that cyclosporine A exerted its effect through inhibition of the mitochondrial permeability transition pore *(159).* More recently, a double-mutant ALS mouse model with a permeable blood–brain barrier was treated with cyclosporine A by intraperitoneal injections, and survival was improved *(160).*

Mitochondria: Trigger or Target in Amyotrophic Lateral Sclerosis?

Although the involvement of mitochondria in the pathogenesis of FALS and SALS is evident, the lack of understanding of the primary cause of motor neuron degeneration makes it difficult to determine whether the role of the mitochondrial dysfunction in the cascade of events leading to motor neuron demise is primary or secondary (Fig. 1).

Mitochondria could be a trigger if the process begins with inhibition of mitochondrial function by protein aggregates. Recent evidence supporting this scenario includes the presence of mutant SOD1 forming aggregates either inside *(73)* or outside *(74,75)* the organelle. Mitochondria could also be a trigger if the primary cause affects mitochondrial function, which would both impair the Ca^{2+}-buffering capacity and increase ROS production *(47,126,161).* On the other hand, the mitochondrial abnormalities could be a target of the ongoing neuronal degeneration, which is initiated somewhere else in the cell. Excitotoxicity associated with EAAT2 *(110,112,117)* or neurofilament abnormalities *(133,134)* could lead to mitochondria changes.

A combination of these two scenarios may occur, because even if they are secondarily damaged, the mitochondria can lead to faster release of cytochrome-*c,* which would hasten apoptosis (Fig. 1). Therefore, mitochondrial dysfunction could have a role in upstream events predisposing to neurodegeneration and in downstream events accelerating neuronal dysfunction and loss *(162).* The available evidence suggests that mitochondrial damage is a critical event in the motor neuron degeneration—at least in the SOD1 mouse models of ALS. Until the detailed mechanisms causing ALS are better understood, this discussion is likely to continue, because mitochondria perform multiple and vital functions in motor neurons.

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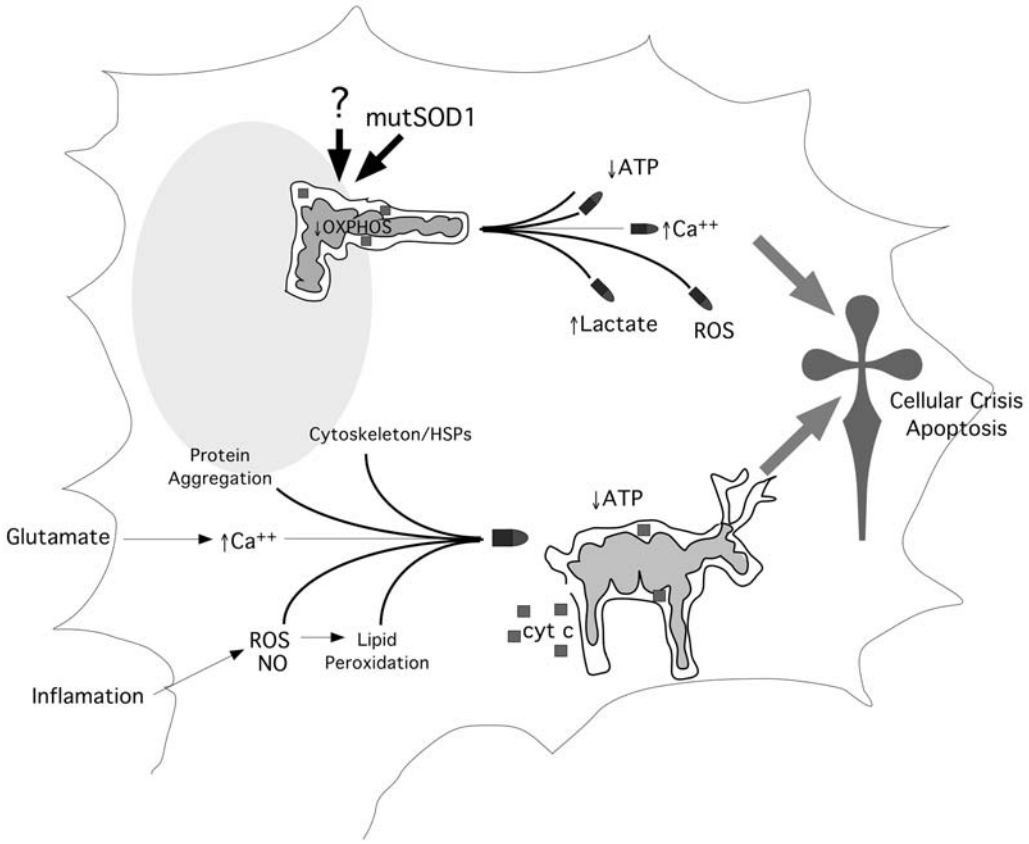

Fig. 1. Mitochondria in ALS: trigger, target, or both? There is no argument about the presence of marked mitochondrial abnormalities in the CNS of humans or animal models with ALS. However, the primary role of these abnormalities is the subject of much discussion. The upper part of the cartoon illustrates how mitochondria could act as a trigger of apoptosis once damaged by mutant SOD1 or other ALS-causing factors (?). In such cases, the cellular levels of adenosine triphosphate decrease, the lost Ca^{2+}-buffering capacity induces higher Ca^{2+} in the cytosol, and increased ROS levels produced by the damaged mitochondria affect other cellular components. On the other hand, mitochondrial abnormalities could be a consequence of ongoing motor neuron degeneration and apoptosis, as illustrated in the lower part of the cartoon. Excitotoxicity-mediated increases in cytosolic Ca^{2+} could promote the opening of the mitochondrial permeability transition pore. Inflammation mediated by astrocytes and microglia could lead to oxidative stress (by ROS and NO) and motor neuron damage. Similarly, protein aggregation and cytoskeleton alterations could restrict mitochondrial movement. All these events would cause mitochondrial apoptotic changes and the release of cytochrome-*c* from the intermembrane space.

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