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## The Mitochondrial Permeability Transition Pore: A Molecular Target for Amyotrophic Lateral Sclerosis Therapy

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**Abbreviated title:** Mitochondria as targets for ALS therapy

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**Abstract**

Effective therapies are needed for the treatment of amyotrophic lateral sclerosis (ALS), a fatal type of motor neuron disease. Morphological, biochemical, molecular genetic, and cell/animal model studies suggest that mitochondria have potentially diverse roles in neurodegenerative disease mechanisms and neuronal cell death. In human ALS, abnormalities have been found in mitochondrial structure, mitochondrial respiratory chain enzymes, and mitochondrial cell death proteins indicative of some non-classical form of programmed cell death. Mouse models of ALS are beginning to reveal possible principles governing the biology of selective neuronal vulnerability that implicate mitochondria. This minireview summarizes work on the how malfunctioning mitochondria might contribute to neuronal death in ALS through the biophysical entity called the mitochondrial permeability pore (mPTP). The major protein components of the mPTP are enriched in mouse motor neurons. Early in the course of disease in ALS mice expressing human mutant superoxide dismutase-1, mitochondria in motor neurons undergo trafficking abnormalities and dramatic remodeling resulting in the formation of mega-mitochondria and coinciding with increased protein carbonyl formation and nitration of mPTP components. The genetic deletion of a major mPTP component, cyclophilin D, has robust effects in ALS mice by delaying disease onset and extending survival. Thus, attention should be directed to the mPTP as rational target for the development of drugs designed to treat ALS.

**Keywords:** adenine nucleotide translocator, apoptosis, cell death, cyclophilin D, excitotoxicity, mitochondria, motor neuron, ppif, voltage-dependent anion channel

## 1. Introduction

ALS is a progressive and severely disabling fatal neurological disease in humans characterized by initial muscle weakness, and then muscle atrophy, spasticity, and eventual paralysis and death typically within 3 to 5 years after symptoms begin [1]. The cause of the spasticity, paralysis and death is progressive degeneration and elimination of upper motor neurons (MNs) in cerebral cortex and lower MNs in brainstem and spinal cord [1,2]. Degeneration and loss of spinal and neocortical interneurons has also been found in human ALS [3,4]. More than 5000 people in the United States are diagnosed with ALS each year (ALS Association, [www.alsa.org](http://www.alsa.org)), and, in parts of the United Kingdom, three people die everyday from some form of MN disease ([www.uk-mnd-professional-network.com](http://www.uk-mnd-professional-network.com)). Other than life support management, no effective treatments exist for ALS [5].

It is still not understood why specific neuronal populations are selectively vulnerable in ALS, such as certain somatic MNs and interneurons [3,4]. The molecular pathogenesis of ALS is understood poorly, contributing to the lack of appropriate target identification and effective mechanism-based therapies to treat this disease. Two forms of ALS exist: idiopathic (sporadic) and heritable (familial). The majority of ALS cases are sporadic with few known genetic contributions, except for missense mutations in TAR-DNA binding protein [6]. Aging is a strong risk factor for ALS because the average age of onset is 55 (ALS Association, [www.alsa.org](http://www.alsa.org)). Familial forms of ALS (fALS) have autosomal dominant or autosomal recessive inheritance patterns and make up ~10% or less of all ALS cases. ALS-linked mutations occur in the genes encoding SOD1 (*ALS1*), Alsin (*ALS2*), senataxin (*ALS4*), vesicle associated membrane protein (VAMP/synaptobrevin)-associated protein B (*ALS8*), dynactin, TAR-DNA binding protein, and fused in sarcoma (*FUS*, *ALS6*) [7,8].

Mitochondrial perturbations have been known for a long time to participate in the mechanisms of neuropathology, particularly disorders involving acute interruptions in O<sub>2</sub> and substrate delivery to the brain and bioenergetic failure as seen in tissue ischemia [9,10]. An exciting new understanding of

mitochondrial biology has emerged over the past decade that is likely to be very relevant to age-related neurodegenerative disorders [7]. Mitochondria are multi-functional organelles [10]. In addition to their critical role in the production of ATP through the electron transport chain (Fig. 1), these organelles function in intracellular  $\text{Ca}^{2+}$  homeostasis, steroid, heme and iron-sulfur cluster synthesis, and programmed cell death [10,11]. They are also sites of formation of reactive oxygen species (ROS), including superoxide anion ( $\text{O}_2^{\bullet-}$ ) and the highly reactive hydroxyl radical ( $\text{OH}^{\bullet}$ ) or its intermediates [10], and reactive nitrogen species such as nitric oxide ( $\text{NO}^{\bullet}$ ) [10]. Thus, mitochondria have functions and properties that might confer an intrinsic susceptibility of subsets of long-lived post-mitotic cells such as neurons to aging and stress. In this regard varying degrees of mitochondrial dysfunction and aberrant mitochondrial maintenance and repair could be critical determinants in the regulation of disease and neuronal cell death ranging from necrosis and apoptosis to autophagy [5,12-14].

## 2. Mitochondrial abnormalities in human ALS

### 2.1. Evidence for mitochondrial abnormalities in human ALS pathogenesis lacks definite causal relationships

Mitochondrial dysfunction has been implicated in the pathogenesis of ALS in humans. Electron microscopy studies have shown mitochondrial morphology abnormalities in skeletal muscle, liver, spinal MNs and cortical upper MN regions of ALS patients [15,16]. A mutation in cytochrome *c* oxidase subunit I was found in a patient with a MN disease phenotype [17]. Another patient with MN disease had a mutation in a mitochondrial tRNA gene [18]. One type of mitochondrial DNA (mtDNA) mutation, called the common mtDNA deletion (mtDNA4977), is found non-uniformly within different human brain areas; the highest levels are detected in the striatum and substantia nigra [19,20]. However, no significant accumulation of the 5kb common deletion in mtDNA has been found by

single-cell analysis of MNs from sporadic ALS cases [21]. Some ALS patients with defects in mitochondrial oxidative phosphorylation in skeletal muscle have a novel SOD1 mutation [22].

## *2.2. Intracellular Ca<sup>2+</sup> abnormalities and excitotoxicity in human ALS pathogenesis: links to mitochondrial dysfunction and oxidative stress*

Mitochondria function in the regulation of intracellular Ca<sup>2+</sup> levels [11,23]. Regarding ALS, skeletal muscle biopsies of patients with sporadic disease show ultrastructural changes indicative of elevated Ca<sup>2+</sup> in MN terminals, with some mitochondria showing an augmented Ca<sup>2+</sup> signal [24]. Utilizing specific transport systems mitochondria can move Ca<sup>2+</sup> from the cytosol into the matrix by the Ca<sup>2+</sup> uniporter and eject Ca<sup>2+</sup> via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger [11] and more catastrophically through the mitochondrial permeability transition pore (mPTP) [25]. Under conditions of elevated cytoplasmic Ca<sup>2+</sup>, whenever the local free Ca<sup>2+</sup> concentration rises above a set-point of ~0.5 μM, mitochondria avidly accumulate Ca<sup>2+</sup> to a fixed capacity [11]. The electrical gradient across the mitochondrial inner membrane, the  $\Delta\Psi_m$ , established by electron transport chain activity (Fig. 1), provides the driving force for the accumulation of Ca<sup>2+</sup> into the mitochondrial matrix [23]. Cytosolic Ca<sup>2+</sup> concentrations above set-point levels are believed to be achieved during tetanic stimulation and by activation of glutamate receptors on the plasma membrane [11]. In settings of the pathological process called excitotoxicity, resulting from excessive overstimulation of glutamate receptors [26], Ca<sup>2+</sup> overload in neurons is significant and cause cell death [27]. When mitochondria become overloaded with Ca<sup>2+</sup>, they undergo mitochondrial permeability transition (see below) resulting in osmotic swelling and rupture of the outer mitochondrial membrane (Fig. 1). Interestingly, mitochondria within synapses appear to be more susceptible than non-synaptic mitochondria to Ca<sup>2+</sup> overload [28].

Excitotoxicity has been implicated in the pathogenesis of ALS for a long time [29] and is another possible mechanism by which MNs can be damaged in ALS [27]. Sporadic ALS patients have

reduced levels of synaptosomal high-affinity glutamate uptake [29] and astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2 or GLT1) in motor cortex and spinal cord [30]. Reductions in levels of activity of EAAT2 in spinal cord could increase the extracellular concentrations of glutamate at synapses on MNs. MNs are sensitive to glutamate excitotoxicity because they have a low proportion of GluR2-edited or under-edited AMPA subtype glutamate receptor on their surfaces, predisposing MNs to risk of excess  $\text{Ca}^{2+}$  entry and mitochondrial perturbations [31,32]. Excess glutamate receptor activation in neurons can cause increased intracellular  $\text{Ca}^{2+}$ , mitochondrial ROS production, bioenergetic failure, and mitochondrial trafficking abnormalities [33].  $\text{Ca}^{2+}$ -induced generation of ROS in brain mitochondria is mediated by mitochondrial permeability transition [34]. MNs are particularly affected by inhibition of mitochondrial metabolism which causes elevated cytosolic  $\text{Ca}^{2+}$  levels and increased excitability [35].

Mitochondria generate endogenous ROS as by-products of oxidative phosphorylation (Fig. 1) [36]. Because many mitochondrial proteins possess iron-sulfur clusters for oxidation-reduction reactions and because mtDNA lacks protective histones, these macromolecules are particularly vulnerable to ROS attack. Electrons in the electron carriers, such as the unpaired electron of ubisemiquinone bound to coenzyme Q binding sites of complexes I, II, and III, can be donated directly to  $\text{O}_2$  to generate  $\text{O}_2^{\bullet-}$  [36].  $\text{O}_2^{\bullet-}$  does not easily pass through biological membranes and thus must be inactivated in compartments where it is generated [37]. The mitochondrial matrix enzyme manganese superoxide dismutase (MnSOD or SOD2) or copper/zinc SOD (Cu/ZnSOD or SOD1) in the mitochondrial intermembrane space and cytosol convert  $\text{O}_2^{\bullet-}$  to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the reaction  $\text{O}_2^{\bullet-} + \text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$  (Fig. 1) [37].  $\text{H}_2\text{O}_2$  is more stable than  $\text{O}_2^{\bullet-}$  and can diffuse from mitochondria and into the cytosol and nucleus.  $\text{H}_2\text{O}_2$  is detoxified by glutathione peroxidase in mitochondria and in the cytosol and by catalase in peroxisomes. In the presence of reduced transitional metals ( $\text{Fe}^{2+}$ ),  $\text{H}_2\text{O}_2$  is catalyzed to  $\text{OH}^{\bullet}$  [38].  $\text{O}_2^{\bullet-}$  can also react with  $\text{NO}^{\bullet}$  to form the potent



nucleophile and oxidant and nitrating agent peroxynitrite ( $\text{ONOO}^-$ ) (Fig. 1) [39].  $\text{ONOO}^-$  or products of  $\text{ONOO}^-$  can damage proteins by nitration [39].  $\text{ONOO}^-$  is genotoxic directly to neurons by causing single- and double-strand breaks in DNA [40]. NO can be produced in mitochondria [41] and has direct effects in mitochondria. NO at nanomolar concentrations can inhibit rapidly and reversibly respiration [42].

Markers of oxidative stress and ROS damage are elevated in ALS tissues [43]. In human sporadic ALS, protein carbonyls are elevated in motor cortex [44]. Tyrosine nitration is elevated in human ALS nervous tissues [45-47]. Studies of respiratory chain enzyme activities are discrepant. Studies have reported increases in complex I, II, and III activities in vulnerable and non-vulnerable brain regions in patients with mutant SOD1-fALS [48], but other studies have found decreased complex IV activity in spinal cord ventral horn [49] and skeletal muscle [50] of sporadic ALS cases. In sporadic ALS skeletal muscle, reductions in activity of respiratory chain complexes with subunits encoded by the mitochondrial genome are associated with decreased neuronal NO synthase levels [51]. Alterations in skeletal muscle mitochondria are progressive [52] and could be intrinsic to skeletal muscle [53] and not due merely to neurogenic atrophy as assumed commonly.

### **3. Human ALS and mitochondrial orchestrated programmed cell death (PCD) involving p53**

#### *3.1. Degeneration of motor neurons in human ALS has features of PCD*

PCD is physiologically regulated cell death [12]. Apoptosis, a form of PCD, is a structurally and biochemically organized, transcriptionally-dependent or –independent, form of cell death. The basic machinery of apoptosis is conserved in yeast, hydra, nematode, fruit fly, zebrafish, mouse, and human [54]. Mitochondria regulate cell death processes (Figs. 1, 2). A variety of mitochondrial proteins function in apoptosis (Table 1) including Bcl-2 family members, cytochrome *c*, apoptosis inducing factor (AIF), endonuclease G, second mitochondrial activator of caspases (Smac/DIABLO),

and Omi/high-temperature requirement protein A2 (HtrA2) inhibitor of the inhibitors of apoptosis proteins (Fig. 1) [55-57]. Other proteins (e.g., humanin, Ku70, 14-3-3 proteins) that are not mitochondrial can modulate mitochondrially translocated cell death proteins by binding and sequestration. For example, Ku70 blocks the translocation of Bax to mitochondria to restrain cell death [58].

PCD appears to contribute to the selective degeneration of MNs in human sporadic ALS and fALS, albeit seemingly as a non-classical form differing from apoptosis (Fig. 2) [59]. MNs appear to pass through sequential stages of chromatolysis (suggestive of initial axonal injury), somatodendritic attrition without extensive cytoplasmic vacuolation, and then nuclear DNA fragmentation, nuclear condensation, and cell death (Fig. 3) [59]. MNs in individuals dying from sporadic ALS and fALS show the same patterns of degeneration [59]. This cell death in human MNs is defined clearly by genomic DNA fragmentation (determined by DNA agarose gel electrophoresis and in situ DNA nick-end labeling) and cell loss and is associated with accumulation of mitochondria, cytochrome *c*, and cleaved caspase-3 (Figs. 2,3) [60]. However, the morphology of this cell death is distinct from classical apoptosis, despite the nuclear condensation [12,61]. Nevertheless, Bax and Bak1 protein levels are increased in mitochondria-enriched fractions of selectively vulnerable motor regions (spinal cord anterior horn and motor cortex gray matter), but not in regions unaffected by the disease (somatosensory cortex gray matter). In marked contrast, Bcl-2 protein is severely depleted in mitochondria-enriched fractions of affected regions and is sequestered in the cytosol (Fig. 2) [59]. Although these western blot observations lacked direct specificity for MN events [59], subsequent immunohistochemistry (Figs. 2,3) [60] and laser capture microdissection of MNs combined with mass spectroscopy-protein profiling have confirmed the presence of intact active caspase-3 in human ALS MNs [62].

### 3.2. Degeneration of motor neurons in human ALS is associated with p53 activation

Neuronal apoptosis can be driven by the tumor suppressor p53 [63]. This cell death can be transcriptionally-dependent and transcriptionally-independent [63]. p53 can mediate mitochondrial permeabilization through direct physical interaction with Bcl-2 family members [64]. p53 is activated in MNs in human ALS [65]. p53 levels increase in vulnerable regions in individuals ALS, and p53 accumulates specifically in ALS MN [65]. This p53 is active functionally because it is phosphorylated at serine<sup>392</sup> and has increased DNA binding activity [61,65].

These data support the concept of an aberrant re-emergence of a PCD mechanism, involving p53 activation and cytosol-to-mitochondria redistributions of cell death proteins, participating in the pathogenesis of MN degeneration in human ALS [59,95]. The morphological and biochemical changes seen in human ALS are modeled robustly and faithfully at structural and molecular levels in axotomy models of MN degeneration in adult mouse [66] but not in the current commonly used human mutant SOD1 transgenic mouse models (Fig. 3) [60].

## 4. Mitochondrial pathobiology in cell and mouse models of ALS

A common mutation in human SOD1 that is linked to fALS is the substitution of glycine by alanine at position 93 (G93A) [67,68]. SOD1 is a metalloenzyme of 153 amino acids (~16 kDa) that binds one copper ion and one zinc ion per subunit [37]. This enzyme, functioning as a ~32 kDa non-covalently linked homodimer, is responsible for the detoxification and maintenance of intracellular  $O_2^{\bullet-}$  concentration in the low femtomolar range by catalyzing its dismutation [37,69]. SOD1 is ubiquitous (intracellular SOD concentrations are typically ~10-40  $\mu$ M) in most tissues and possibly greater in neurons [70].

#### *4.1. Evidence for mitochondrial abnormalities in cell models of ALS*

Data from cell culture studies argue strongly for mitochondria dysfunction, possibly MN selective, in the presence of mSOD1 [67,71]. Expression of several human SOD1 mutants increases mitochondrial  $O_2^{\bullet-}$  levels and causes toxicity in primary MNs [72], neuroblastoma cells [73], and NSC-34 cells (a hybrid cell line with some MN-like characteristics, produced by fusion of MN-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells) [75]. These responses can be attenuated by over-expression of MnSOD [73]. ALS-mSOD1 variants, compared to wild-type SOD1, associate more with mitochondria in NSC-34 cells and appear to form cross-linked oligomers that shift the mitochondrial GSH/GSSH ratio toward oxidation [71].

#### *4.2. Most evidence for mitochondrial abnormalities in mouse ALS pathogenesis lacks definite causal relationships*

Gurney et al were the first to develop transgenic (tg) mice that express the G93A mutant form of human SOD1 [76,77]. Now, these mice are used widely as an animal model of ALS [5,60,67,68]. Human mutant SOD1 (mSOD1) is expressed ubiquitously in these mice by its endogenous promoter in a tissue/cell non-selective pattern against a background of normal mouse SOD1 [76]. Effects of this human mutant gene in mice are profound. Hemizygous tg mice expressing high copy number of the G93A variant of mSOD1 become completely paralyzed and die at ~16-18 weeks of age [76]. G93A-mSOD1 mice with reduced transgene copy number have a much slower disease progression and die at ~8-9 months of age [76,78]. Spinal MNs and interneurons in mice expressing G93A<sup>high</sup>-mSOD1 undergo prominent degeneration; about 70% of lumbar MNs are eliminated by end-stage disease [79,80]. More work is needed on the cell death and its mechanisms in G93A<sup>low</sup>-expressing mice. Subsets of spinal interneurons are lost before MNs G93A<sup>high</sup>-mSOD1 [79], some of which are the

glycinergic Renshaw cells [80]. Unlike the degeneration of MN in human ALS, MNs in these mice do not degenerate with a morphology resembling any form of apoptosis (Fig. 3) [60,79,81]. The MN degeneration seen in G93A<sup>high</sup>-mSOD1 mice more closely resembles a prolonged necrotic-like cell death process (Fig. 3) [12] involving early-occurring mitochondrial damage, cellular swelling, and dissolution [60,78-80]. Biochemically, the death of MNs is characterized by somal and mitochondrial swelling and formation of DNA single-strand breaks prior to double-strand breaks occurring in nuclear DNA and mitochondrial DNA [79]. The MN death is independent of activation of caspases-1 and 3, and also appears to be independent of caspase-8 and apoptosis-inducing factor activation within MNs [79]. Indeed, caspase-dependent and p53-mediated apoptosis mechanisms might be blocked actively in G93A<sup>high</sup>-mSOD1 mouse MNs, possibly by upregulation of inhibitors of apoptosis and changes nuclear import of proteins [79].

Mitochondrial pathology has been implicated in the mechanisms of human and mouse ALS [7], but most evidence is circumstantial. In different mSOD1 mouse models of ALS, mitochondria in spinal cord neurons exhibit structural pathology [79,82-86] and some of the mitochondrial degeneration occurs very early in the course of the disease [79,81]. Mitochondrial microvacuolar damage in MNs emerges by 4 weeks of age in G93A mice with high expression [79,81]. It has been argued that mitochondrial damage in G93A<sup>high</sup>-mSOD1 mice is related to supra-normal levels of SOD1 and might not be related causally to the disease process because transgenic mice expressing high levels of human wildtype SOD1 show some mitochondrial pathology [87], but mitochondrial abnormalities have been found histologically also in G93A<sup>low</sup>-mSOD1 mice [88](Martin LJ et al, unpublished observations). Thus, mitochondria could be primary sites of human SOD1 toxicity in transgenic mice irrespective of transgene copy number and expression level of human SOD1, but direct, unequivocal causal relationships have been lacking.

Human SOD1 mutant proteins appear to gain a toxic property or function, rather than having diminished  $O_2^-$  scavenging activity [89-91], and wild-type SOD1 can gain toxic properties through oxidative modification [92,93]. A gain in aberrant oxidative chemistry could contribute to the mechanisms of mitochondriopathy in G93A<sup>high</sup> mice [39,94]. G93A-mSOD1 has enhanced free radical-generating capacity compared to wild-type enzyme [91] and can catalyze protein oxidation by hydroxyl-like intermediates and carbonate radical [95]. G93A<sup>high</sup> mice have increased protein carbonyl formation in total spinal cord tissue extracts at pre-symptomatic disease [96]. Protein carbonyl formation in mitochondrial membrane-enriched fractions of spinal cord is a robust signature of incipient disease [78]. A mass spectroscopy study of G93A<sup>high</sup> mice identified proteins in total spinal cord tissue extracts with greater than baseline carbonyl modification, including SOD1, translationally controlled tumor protein, and ubiquitin carboxyl-terminal hydrolase-L1 [97]. Nitrated and aggregated cytochrome *c* oxidase subunit-I and  $\alpha$ -synuclein accumulate in G93A<sup>high</sup> mouse spinal cord [79]. Nitrated MnSOD accumulates also in G93A<sup>high</sup> mouse spinal cord [79]. Toxic properties of mSOD1 could also be mediated through protein binding or aggregation. Wild-type SOD1 and human mSOD1 associate with mitochondria [85,98]. Human SOD1 mutants associate with spinal cord mitochondria in mSOD1 mice and can bind Bcl-2 [99,100], thus potentially being decoys or dominant negative regulators of cell survival molecules (Fig. 2), but it is not known if this process is occurring specifically in mouse MNs. Bcl-2 is depleted from mitochondria isolated from micropunches of human ALS anterior horn gray matter and accumulates in the cytosol (Fig. 2) [59]. Binding of mSOD1 (and perhaps its low-mobility species) to mitochondria has been reported to be spinal cord selective and age-dependent [101], but this work lacks cellular resolution. A recent biochemical in vitro study has shown that endogenous SOD1 in the mitochondrial intermembrane space controls cytochrome *c*-catalyzed peroxidation and that G93A-mSOD1 mediates greater ROS production in the intermembrane space compared to wild-type SOD1 [102]. Human SOD1 mutants can also shift

mitochondrial redox potential when expressed in cultured cells [71]. Nevertheless, the direct links between the physicochemical changes in mSOD1 and wild-type SOD1 and the mitochondrial functional and structural changes associated with ALS and MN degeneration remain uncertain.

EM studies have shown that the outer mitochondrial membrane (OMM) remains relatively intact to permit formation of mega-mitochondria in MNs cell bodies in G93A<sup>high</sup> mice [78,79]. Moreover, early in the disease of these mice, mitochondria in dendrites in spinal cord ventral horn undergo extensive cristae and matrix remodeling, while few mitochondria in MN cell bodies show major structural changes [78]. Another interpretation of ultrastructural findings is that the mSOD1 causes mitochondrial degeneration by inducing OMM extension and leakage and intermembrane space expansion [86]. Mechanisms for this damage could be related to mSOD1 gaining access to the mitochondrial intermembrane space [83,102] and the matrix [103] and inducing disturbances in oxidative phosphorylation [104] and antioxidant activity. This mitochondrial conformation seen by EM might favor the formation of the mPTP (Fig. 1); indeed, we found evidence for increased contact sites between the OMM and inner mitochondrial membrane (IMM) in dendritic mitochondria in G93A<sup>high</sup> mice [78]. Another feature of MNs of young G93A<sup>high</sup> mice before symptoms is apparent fission of ultrastructurally normal mitochondria in cell bodies and fragmentation of abnormal mitochondria [78]. It is not clear if the cristae and matrix remodeling and the apparent fragmentation and fission mitochondria are related or independent events and if these abnormalities interfere with mitochondrial trafficking; nevertheless, morphological observations enforce the idea that mitochondria are critical to the pathobiology of mSOD1 toxicity to MNs in G93A<sup>high</sup> mice.

The possibility of changes in mitochondrial trafficking in MNs of mSOD1 mice is mostly unexplored. Some data support the novel idea that mitochondria might act as messengers from distal regions of MNs in mSOD1 mice [7]. G93A<sup>high</sup>-mSOD1 mouse MNs accumulate mitochondria from the axon terminals and generate higher levels of O<sub>2</sub><sup>•-</sup>, <sup>•</sup>NO, and ONOO<sup>-</sup> than MNs in transgenic mice

expressing human wild-type SOD1 [79]. This mitochondrial accumulation occurs at a time when MN cell body volume is increasing, suggestive of ongoing problems with ATP production or plasma membrane Na,K ATPase [79]. G93A-mSOD1 perturbs anterograde axonal transport of mitochondria in cultured primary embryonic MNs [105] making it possible that retrogradely transported mitochondria with toxic properties from the neuromuscular junction fail to be returned to distal processes [7,79]. Mitochondria with enhanced toxic potential from distal axons and terminals could therefore have a “Trojan horse” role in triggering degeneration of MNs in ALS via retrograde transport from diseased skeletal muscle.

MNs in G93A<sup>high</sup>-mSOD1 mice also accumulate higher levels of intracellular Ca<sup>2+</sup> than MNs in transgenic human wild-type SOD1 mice [79]. The intracellular Ca<sup>2+</sup> signal in MNs is very compartmental and mitochondrial-like in its appearance [79,106]. Abnormal elevations intracellular Ca<sup>2+</sup> in G93A<sup>high</sup>-mSOD1 mouse MNs have been seen also by different Ca<sup>2+</sup> detection methods [107,108]. Brain and spinal cord mitochondria in G93A mice handle Ca<sup>2+</sup> abnormally and have impaired Ca<sup>2+</sup> uptake capacity [109], but this work lacks cellular resolution. However, recent elegant work using a mouse neuromuscular junction preparation has revealed that, specifically within MN terminals, mitochondrial Ca<sup>2+</sup> accumulation is accompanied by greater IMM depolarization in human mutant SOD1 transgenic mice [110].

NO signaling mechanisms in mitochondria of ALS mice have also been implicated in the pathogenesis (Fig. 1). MNs seem to be unique regarding NO production because they express constitutively low levels of inducible NO synthase (iNOS) [106]. G93A<sup>high</sup>-mSOD1 mouse MNs accumulate nicotinamide adenine dinucleotide phosphate diaphorase and iNOS-like immunoreactivity [79]. iNOS is also up-regulated aberrantly in human sporadic ALS MNs [111]. *iNOS* gene deletion extends significantly the lifespan of G93A<sup>high</sup>-mSOD1 mice [79]. Thus, mitochondrial oxidative stress, Ca<sup>2+</sup> dysregulation, iNOS activation, protein nitration, and protein aggregation (not necessarily SOD1



though) are all likely intrinsic, cell autonomous mechanisms in the process of MN degeneration caused by mSOD1 in mice [79]. The mechanistic basis for the differences between human ALS and mSOD1 mice, regarding cell death phenotype (Fig. 3), is not yet clear but could be related to the supra-normal expression of toxic mSOD1 or to fundamental differences in cell death mechanisms [60] or the tissue inflammatory milieu (Martin LJ, unpublished observations) that drive MNs in mSOD1 transgenic mice to necrotic-like death along the apoptosis-necrosis cell death continuum [12,13,106]. Another contributing factor for this difference between human and mouse MNs is that mitochondria are functionally diverse and have species-specific activities and molecular compositions, including the makeup of the mitochondrial permeability transition pore [112]. These possibilities allow for skepticism regarding the suitability of existing transgenic mSOD1 mouse lines to model human ALS.

## **5. The mitochondrial permeability transition pore contributes to the causal mechanisms of ALS in mice**

Despite the implication of toxic effects of mSOD1 on mitochondria in mouse ALS, cause-effect relationships between abnormal functioning of mitochondria and initiation and progression of disease have been uncertain. These relationships need to be known because this knowledge could provide a rationale for new mechanism-based treatments for ALS. One venue of investigation for mitochondrial damage causality is the mPTP and its possible involvement in ALS.

### *5.1. The mPTP: definition and molecular composition*

Mitochondrial permeability transition is a mitochondrial state in which the proton-motive force is disrupted reversibly or irreversibly [25,76,113-117]. Conditions of mitochondrial  $\text{Ca}^{2+}$  overload, excessive oxidative stress, and decreased electrochemical gradient ( $\Delta P$ ), ADP, and ATP can favor mitochondrial permeability transition. This alter state of mitochondria involves the mPTP that functions

as a voltage, thiol, and  $\text{Ca}^{2+}$  sensor [25,76,113-117]. The mPTP is believed to be a poly-protein transmembrane channel formed at the contact sites between the IMM and the OMM. The collective components of the mPTP are still controversial, but the voltage-gated anion channel (VDAC, or porin) in the OMM, the adenine nucleotide translocator (ANT, or solute carrier family 25) in the IMM, and cyclophilin D (CyPD) in the matrix are believed to be the core components (Table 1) [114-116]. Other components or modulators of the mPTP appear to be hexokinase, creatine kinase, translocator protein 18 kDa (TSPO, or peripheral benzodiazepine receptor), and Bcl-2 family members (Table 1) [113-117].

The VDAC family in human and mouse consists of three proteins of ~31 kDa (VDAC1-3) encoded by three different genes [118]. VDACS are the major transport proteins in the OMM, functioning in ATP rationing,  $\text{Ca}^{2+}$  homeostasis, oxidative stress response, and apoptosis [118]. Monomeric VDAC serves as the functional channel, although oligomerization of VDAC into dimers and tetramers can occur and might function in cell death [118]. The VDAC adopts an open conformation at low or zero membrane potentials and a closed conformation at potentials above 30-40 mV making the OMM permeable to most small hydrophilic molecules up to 1.3 kDa for free exchange of respiratory chain substrates [119]. Most data implicating VDAC opening or closing as an important regulator of cell death are based on in vitro conditions, while limited in vivo evidence is available [120]. VDAC1 binds Bak1, hexokinase, gelsolin, and ANT1/ANT2; VDAC2 binds Bak1, hexokinase, cytochrome c, glycerol kinase, and ANT1/ANT2; VDAC3 binds glycerol kinase, CyPD, and ANT1-3 [118]. In human tissues, VDAC1 and VDAC2 isoforms are expressed more abundantly than VDAC3; highest levels are found in kidney, heart, skeletal muscle, and brain [121]. The effects of selective knockout of VDAC isoforms are not equivalent, implying different functions. Mice deficient in either VDAC1 or VDAC3 are viable [122-124], but VDAC2 deficiency causes embryonic lethality [125]. Lack of both VDAC1 and VDAC3 causes growth retardation [124]. VDAC null mouse tissues exhibit deficits in mitochondrial respiration

and abnormalities in mitochondrial ultrastructure [122]. Mitochondria without VDAC1 have an intact mitochondrial permeability transition response [126,127]. VDAC2 deletion, but not lack of the more abundant VDAC1, results in enhanced activation the mitochondrial apoptosis pathway and enforced activation of Bak at mitochondria [125], consistent with the idea that VDAC2 is a key inhibitor of Bak-mediated apoptosis [124]. However, other data show that cells lacking individual VDACS or combinations of VDACS have normal death responses to Bax and Bid [127]. New work in yeast has revealed that SOD1 is necessary for proper functioning of VDAC; specifically, SOD1 regulates VDAC channel activity and protein levels in mitochondria [128].

The mitochondrial ANT family in human consists of 3 members (ANT1-3, or solute carrier family 25, members 4, 5, and 6) encoded by three different genes, but in mouse only two isoforms of the ANT are present [128]. The proteins are ~33 kDa and function as homodimers [128]. They are multi-pass membrane proteins, with odd-numbered transmembrane helices having kinks because of proline residues, which mediate exchange of cytosolic ADP for mitochondrial ATP across the inner membrane utilizing the electrochemical gradient [129]. ANT1 binds VDAC1, CyPD, Bax, twinkle (ataxin-8), and cyclophilin-40; ANT2 binds VDAC1-3 and cyclophilin-40; ANT3 binds VDAC1, steroid sulfatase, and translocase of inner mitochondrial membrane 13 and 23 [128]. The ANT isoforms are expressed differentially in tissue- and species-specific patterns [130]. ANT1 is expressed highly in human and mouse heart and skeletal muscle, and human brain has low ANT1 mRNA but high ANT3 mRNA, while mouse brain has high ANT1 mRNA [130]. ANT2 mRNA is very low or not expressed in most adult human and mouse tissues, with kidney having some expression [130]. In tissue mitochondria where more than one ANT is found, it is ANT1 that binds preferentially to CyPD to form the mPTP at contact sites between the inner and outer mitochondrial membranes [131]. It has been proposed that, in the presence of high mitochondrial  $\text{Ca}^{2+}$ , the binding of CyPD to proline residue 61 (Pro<sup>61</sup>) in loop 1 of ANT1 results in a conformation that converts the ANT into a non-specific pore

[128]. Non-conditional ANT1 null mice are viable and grow normally but develop mitochondrial skeletal myopathy and cardiomyopathy [129]. Ablation both ANT isoforms in mouse liver surprisingly did not change fundamentally mitochondrial permeability transition and cell death in hepatocytes [132], and some ANT ligands induce mitochondrial dysfunction and cytochrome *c* release independent of mitochondrial permeability transition [133]. Thus, the mechanisms of ANT-induced cell death are not understood fully.

CyPD (also named cyclophilin F, peptidyl prolyl isomerase F) is encoded by a single gene [114,116,134]. Despite confusing nomenclature, there is only one isoform of CyPD (EC 5.2.1.8, *ppif* gene product) in mouse and human. The ~20 kDa protein encoded by this gene is a member of the peptidyl-prolyl *cis-trans* isomerase (PPIase) family. PPIases catalyze the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins. CyPD binds ANT1 [129].

During normal mitochondrial function the OMM and the IMM are separated by the intermembrane space, and the VDAC and the ANT do not interact [114]. Permeability transition is activated by the formation of the mPTP; the IMM loses its integrity and the ANT changes its conformation from its native state into a non-selective pore [135]. This process is catalyzed by CyPD that functions in protein *cis-trans* isomerization and chaperoning [136]. The ANT and CyPD interact directly [137]. The molar concentration of CyPD (in heart mitochondria) is much less (>5%) than ANT; thus, under normal conditions only a minor fraction of the ANT can be in a complex with CyPD [117,138]. When this occurs, small ions and metabolites permeate freely across the IMM and oxidation of metabolites by O<sub>2</sub> proceeds with electron flux not coupled to proton pumping, resulting in collapse of  $\Delta P$ , dissipation of ATP production, production of ROS, equilibration of ions between the matrix and cytosol, matrix volume increases, and mitochondrial swelling [115,119].

## 5.2. Expression and localization of mPTP components in the CNS

Very few studies have been published on the localizations of mPTP components in the mammalian CNS; thus, details about the cellular expressions in different nervous system cell types are lacking. VDAC expression patterns are complicated by alternative splicing that generates two different VDAC1 mRNAs, three different VDAC2 mRNAs, and two different VDAC3 mRNAs [118]. Studies of nervous tissue have found VDAC in neurons and glial cells [139] and associated with mitochondria, the endoplasmic reticulum, and the plasma membrane [140,141]. Non-mitochondrial localizations of VDAC have been disputed [142]. Information on ANT localizations in nervous tissue is particularly scarce. ANT appears to be expressed in reactive astrocytes [143]. The few existing studies on CyPD localization in mammalian CNS have found it enriched in subsets of neurons in adult rat brain, with some interneurons being positive [144], and relative low levels in astrocytes [145,146].

In mouse spinal cord, the core components of the mPTP (VDAC, ANT, and CyPD) are enriched in MNs as determined by immunohistochemistry [78]. The specific isoforms of ANT and VDAC in MNs have not been determined. CyPD, ANT, and VDAC have mitochondrial and non-mitochondrial localizations in MNs [78]. They are all nuclear-encoded mitochondrial-targeted proteins, thus a possible explanation for their non-mitochondrial localizations is that they are pre-mitochondrial forms. Some cyclophilins are located in the cytoplasm [146], but CyPD immunoreactivity is annulled in *ppif*<sup>-/-</sup> mice, demonstrating that the antibody is detecting only CyPD [78]. Spinal cord, brainstem, and forebrain had similar levels of CyPD, as well as similar levels of ANT and VDAC [78]. Thus, differences in the levels of individual mPTP components cannot explain the intrinsic differences in the sensitivity to Ca<sup>2+</sup>-induced permeability transition seen in spinal cord and brain isolated mitochondria [147, 148]. Not all mitochondria within individual MNs contained CyPD, ANT, and VDAC [78]; this observation supports that idea of that mitochondria in individual cells are not only heterogeneous in shape [149,150] but also in biochemical composition, notably metabolism [151] and genetics [36].

### 5.3. *The mPTP contributes to the pathogenesis of mouse ALS*

The mPTP was first implicated in ALS pathogenesis using pharmacological approaches. Cyclosporine A treatment of G93A<sup>high</sup> mice, delivered intracerebroventricularly or systemically to mice on a multiple drug resistance type 1a/b background, modestly improved outcome [152-154], but these studies are confounded by the immunosuppressant actions of cyclosporine A through calcineurin inhibition. Pharmacological studies using CyPD inhibitors devoid of effects on calcineurin need to be done on ALS mice. Another study showed that treatment with cholest-4-en-3-one oxime (TRO19622), a drug that binds VDAC and the 18 kDa translocator protein (TSPO, or peripheral benzodiazepine receptor), improved motor performance, delayed disease onset, and extended survival of G93A<sup>high</sup> mice [155]. However, another study using a different TSPO ligand (Ro-4864) did not show positive effects with G93A<sup>high</sup> mice [156].

CyPD and ANT have been identified as targets of nitration in ALS mice [78]. CyPD nitration is elevated in early- to mid-symptomatic stages, but declines to baseline at end-stage disease [78]. ANT nitration is notable particularly because it is found in pre-symptomatic and symptomatic stages but not at end-stage disease or in transgenic mice expressing human wild-type SOD1 [78]. The ANT is important in the context of age-related neurodegenerative disease because it undergoes carbonyl modification during aging in housefly flight muscle [157] and rat brain [158]. In vitro cell-free and cell experiments have shown that NO and ONOO<sup>-</sup> can act directly on the ANT to induce mitochondrial permeabilization in a cyclosporine A-sensitive manner [159]. Oxidative stress enhances the binding of CyPD to ANT [160]. Some SOD1 mutants are unstable and loose copper [67], and interestingly, copper interactions with ANT and thiol modification of ANT can cause mPTP opening [161-163]. Together these data and future work could reveal that oxidative and nitrative damage to proteins,

some of which are core components of the mPTP, in G93A<sup>high</sup> mice is targeted rather than stochastic and could impinge on the functioning of the mPTP.

The role of CyPD in the process of MN disease has been examined in ALS mice through gene-ablation [78]. G93A<sup>high</sup>-mSOD1 mice without CyPD show markedly delayed disease onset and lived significantly longer than transgenic mice with CyPD. The effect of CyPD deletion was much more prominent in females than in males [78]. Female mice even showed positive effects with haplo-deletion of CyPD. *Ppif* gene ablation in transgenic mice with much lower levels of human mSOD1 expression and a slower disease progression (G93A<sup>low</sup>-mSOD1 mice) also show significantly delayed disease onset and lived significantly longer than transgenic mice with CyPD [78]. Thus, some form of mitochondrial pathobiology is occurring regardless of whether transgene expression of G93A is high or low.

Nevertheless, G93A-mSOD1 mice without CyPD develop eventually MN disease and die. Other work on CypD null mice has shown that high concentrations of Ca<sup>2+</sup> (2 mM) can still lead to mPTP activation without CyPD and that cell deaths caused by Bid, Bax, DNA damage and TNF- $\alpha$  are not affected [164]. The effects of CyPD deficiency on MN cell mechanisms thus need to be examined in more detail, but the cell death phenotype might be switched or converted to another form with the attenuation of mitochondrial swelling. A switch in the cell death morphology and molecular mechanisms in MNs of mSOD1 mice without CyPD is an outcome consistent with the cell death continuum concept [12].

## 6. Summary and outlook

ALS is the 3<sup>rd</sup> most common human neurodegenerative disease with an adult onset [1,2]. It is a paralytic disease that destroys MNs and skeletal muscle and cannot yet be cured or treated effectively; thus contracting the disease is fatal [5]. Mitochondria have diverse functions and

properties and could be critically important for the development of human ALS [7]. Structural and biochemical data from studies of human ALS and cell/animal models of ALS suggest that mitochondrial dysfunction is a trigger or propagator of neurodegeneration. Mitochondria in subsets of neurons and skeletal muscle cells could be compromised in ALS, rendering these cells intrinsically susceptible to cellular aging and stress. Novel mechanisms for mitochondriopathy and MN degeneration in human and mouse ALS could involve apoptosis, accumulation of intracellular  $\text{Ca}^{2+}$ , abnormal trafficking of mitochondria with enhanced ROS toxic potential from distal dendrites or terminals at the neuromuscular junction, distal axonopathy and target deprivation, NOS trafficking and localization to MN mitochondria, and ONOO<sup>-</sup> damage [79]. The mPTP actively participates in the mechanisms of MN death in ALS mice in a gender-dependent pattern. Thus, mPTP activation is a possible triggering event for MN degeneration and MN selective vulnerability in ALS could be related to amount, composition, and trafficking of mitochondria in MNs and the association of MN terminals with skeletal muscle cells. There is precedence for this logic in mouse models of Alzheimer's disease [165], multiple sclerosis [166], and stroke [167]. Further study of mitochondria in neurons and skeletal myocytes can define new mechanisms of disease in ALS and can lead to the identification of molecular mechanism-based therapies for treating this fatal disease.

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## Figure Legends

**Figure 1. Mitochondrial regulation of cell life and death in schematic representation.** The respiratory chain proteins (complex I-IV) establish an electrochemical gradient across the inner mitochondrial membrane (IMM) by extruding protons out of the matrix into the intermembrane space, thereby creating an energy gradient that drives the production of ATP by complex V (lower left). Superoxide ( $O_2^{\bullet-}$ ) is produced as a by-product in the process of electron transport and is converted to hydrogen peroxide ( $H_2O_2$ ) by MnSOD (or CuZnSOD). In pathological settings that can trigger cell aging and death,  $H_2O_2$  can be converted to hydroxyl radical (OH), or hydroxyl-like intermediates, and mitochondrial nitric oxide synthase (NOS) can produce nitric oxide (NO) that can combine with  $O_2^{\bullet-}$  to form peroxynitrite ( $ONOO^-$ ). CuZnSOD can use  $ONOO^-$  to catalyze the nitration of mitochondrial protein tyrosine residues (bottom center) such as cyclophilin D (CyPD) and the adenine nucleotide translocator (ANT) [78]. Bcl-2 family members regulate apoptosis by modulating the release of cytochrome c from mitochondria into the cytosol. Two models can account for this process (the Bax/Bak channel model and the mitochondrial apoptosis-induced channel or MAC). In the Bax/Bak1 channel model (left), Bax (Bcl-2-associated X protein) is a pro-apoptotic protein found mostly in the cytosol in healthy mammalian cells but, after specific cell death inducing stimuli, Bax undergoes a conformation shift and translocates to the outer mitochondrial membrane (OMM) where it inserts [55,56]. Bak1 (Bcl-2-antagonist/killer 1) is a similar pro-apoptotic protein localized mostly to the mitochondrial outer membrane. Bax/Bak1 monomers physically interact and form oligomeric or heteromeric channels that are permeable to cytochrome c. The formation of these channels is blocked by Bcl-2 and Bcl-x<sub>L</sub> at multiple sites. BH3-only members (Bad, Bid, Noxa, Puma) are pro-apoptotic and can modulate the conformation of Bax/Bak1 to sensitize this channel, possibly by exposing its membrane insertion domain (not shown). The MAC could be a channel similar to the

Bax/Bak1 channel, but it might also have additional components. Release cytochrome *c* participates in the formation of the apoptosome in the cytosol that drives the activation of caspase-3 in motor neurons (see Figure 2). Second mitochondria-derived activator of caspases (Smac)/ direct IAP-binding protein with low pI (DIABLO) are released into the cytosol to inactivate the anti-apoptotic actions of inhibitor of apoptosis proteins that inhibit caspases. The DNases AIF and EndoG are released and translocate to the nucleus to stimulate DNA fragmentation. Another model (right) for mitochondrial directed cell death involves the permeability transition pore (PTP). The PPT is a transmembrane channel formed by the interaction of the ANT and the voltage-dependent anion channel (VDAC) at contact sites between the IMM and the OMM [114-120]. CyPD, located in the matrix, can regulate the opening of the PTP by interacting with the ANT. Opening of the PTP induces matrix swelling and OMM rupture leading to release of cytochrome *c* and other apoptogenic proteins (AIF, EndoG). Certain Bcl-2 family members can modulate the activity of the PTP.

**Figure 2. PCD mechanisms regulate the degeneration of motor neurons in human amyotrophic lateral sclerosis.** The levels of multidomain Bcl-2 family members are aberrant in isolated mitochondria from human amyotrophic lateral sclerosis (ALS) brain and spinal cord [59]. Bcl-2 is depleted from mitochondria by unknown mechanisms (top schematic drawing). Bax and Bak levels are elevated in mitochondria (top schematic drawing). These changes would favor the release of cytochrome *c* from mitochondria and the accumulation of cytochrome *c* in the cytoplasm of motor neurons (A). Human ALS motor neurons are rich in mitochondria, as identified by cytochrome *c* oxidase subunit I (B, brown labeling) and many are positive for cleaved capsase-3 (B, blue-green labeling). These observations are consistent with an accumulation of somatodendritic mitochondria and apoptosome-mediated activation of caspase-3 in ALS motor neurons. These changes appear to be occurring slowly in subsets of motor neurons and could lead to cell death that is a variant phenotype of apoptosis (see Figure 3). In human ALS motor neurons, it is not known yet if alterations

occur in the components of the mPTP (top schematic drawing), such as cyclophilin D (CypD), adenine nucleotide translocator (ANT), and voltage-dependent anion channel (VDAC). Scale bars = 6  $\mu\text{m}$  (A); 3  $\mu\text{m}$  (B).

**Figure 3. Comparison of motor neuron death phenotypes in human and transgenic mouse amyotrophic lateral sclerosis.** The degeneration of spinal motor neuron (MNs) in human fALS and in G93A<sup>high</sup> mSOD1 mouse is very different. **A-C.** Normal anterior horn MNs in human spinal cord are large polygonal multipolar cells as seen with cresyl violet staining (A). In human fALS, the degeneration is typified by shrinkage and progressive condensation of the cytoplasm and nucleus (B,C, \* identifies the nucleus). The cell in (c) is identified as a dying MN based the large nucleolus and residual large Nissl bodies. **D.** These shrunken human MNs usually have large-scale genomic fragmentation of DNA as detected by terminal transferase-mediated biotin-dUTP nick-end labeling (TUNEL). Brown labeling in nucleus (asterisk) reveals the presence of DNA-double strand breaks. **E.** Degenerating MNs in human ALS are always immunopositive for cleaved caspase-3 (black-dark green labeling) in the somatodendritic attrition stage and accumulate around the nucleus (asterisk) discrete mitochondria (brown-orange labeling, detected with antibody to cytochrome c oxidase subunit I) exhibiting little light microscopic evidence for swelling. **F.** Human wild-type SOD1 transgenic mice have mostly normal appearing spinal MNs as seen with cresyl violet staining. **G-I.** In G93A<sup>high</sup> mSOD1mice (G1 line), the degeneration is typified by progressive cytoplasmic vacuolization and cellular swelling and dissolution (\* identifies the nucleus) that begins at about 4-6 week of age develops severely from 8 to 16 weeks of age. This degeneration more closely resembles a form of slowly occurring necrosis. Neither the cytoplasm nor the nucleus undergoes condensation during their degeneration. Scale bars: A (same for B,C) = 8  $\mu\text{m}$ ; D (same for E) = 12  $\mu\text{m}$ ; F (same for G-I) = 10  $\mu\text{m}$ .

<b>Table 1. Some Mitochondrial Associated Proteins That Function in Cell Death</b>	
<b>Protein</b>	<b>Function</b>
Bcl-2*	Antiapoptotic, blocks Bax/Bak channel formation
Bcl-X <sub>L</sub>	Antiapoptotic, blocks Bax/Bak channel formation
Bax*	Proapoptotic, forms pores for cytochrome release
Bak*	Proapoptotic, forms pores for cytochrome release
Bad	Proapoptotic, decoy for Bcl-2/Bcl-X <sub>L</sub> promoting Bax/Bak pore formation
Bid	Proapoptotic, decoy for Bcl-2/Bcl-X <sub>L</sub> promoting Bax/Bak pore formation
Noxa	Proapoptotic, decoy for Bcl-2/Bcl-X <sub>L</sub> promoting Bax/Bak pore formation
Puma	Proapoptotic, decoy for Bcl-2/Bcl-X <sub>L</sub> promoting Bax/Bak pore formation
p53*	Antagonizes activity of Bcl-2/Bcl-X <sub>L</sub> , promotes Bax/Bak oligomerization
Cytochrome c	Activator of apoptosome
Smac/DIABLO	IAP inhibitor
AIF	Antioxidant flavoprotein/released from mitochondria to promote nuclear DNA fragmentation
Endonuclease G	Released from mitochondria to promote nuclear DNA fragmentation
HtrA2/Omi	IAP inhibitor
VDAC	mPTP component in outer mitochondrial membrane
ANT <sup>†</sup>	mPTP component in inner mitochondrial membrane
Cyclophilin D <sup>†</sup>	mPTP component in mitochondrial matrix
TSPO (peripheral benzodiazepine receptor)	Modulator of mPTP
Hexokinase	Modulator of VDAC

\*Known to be changed in human ALS [59,65]

<sup>†</sup>Oxidatively modified in mouse ALS [78]







