MINI REVIEW

Mitochondrial pathobiology in ALS

Lee J. Martin

Abstract Amyotrophic lateral sclerosis (ALS) is the third most common human adult-onset neurodegenerative disease. Some forms of ALS are inherited, and disease-causing genes have been identified. Nevertheless, the mechanisms of neurodegeneration in ALS are unresolved. Genetic, biochemical, and morphological analyses of human ALS as well as cell and animal models of ALS reveal that mitochondria could have roles in this neurodegeneration. The varied functions and properties of mitochondria might render subsets of selectively vulnerable neurons intrinsically susceptible to cellular aging and stress and overlying genetic variations. Changes occur in mitochondrial respiratory chain enzymes and mitochondrial programmed cell death proteins in ALS. Transgenic mouse models of ALS reveal possible principles governing the biology of neurodegeneration that implicate mitochondria and the mitochondrial permeability transition pore. This paper reviews how mitochondrial pathobiology might contribute to the mechanisms of neurodegeneration in ALS.

Keywords Apoptosis · Cyclophilin D · Mitochondrial permeability transition pore · Motor neuron · Voltage-dependent anion channel

Synopsis of human ALS

ALS is a progressive and severely disabling neurological disease in humans characterized by initial muscle weakness,

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and then muscle atrophy, spasticity, and eventual paralysis and death typically 3 to 5 years after diagnosis (Rowland and Shneider 2001). The cause of the spasticity, paralysis and death is progressive degeneration and elimination of upper motor neurons in cerebral cortex and lower motor neurons in brainstem and spinal cord (Fig. 1) (Rowland and Shneider 2001; Sathasivam et al. 2001). Degeneration and loss of spinal and neocortical interneurons also occur in human ALS (Stephens et al. 2006; Maekawa et al. 2004). More than 5,000 people in the USA are diagnosed with ALS each year (ALS Association, http://www.alsa.org), and, in parts of the United Kingdom, three people die every day from some form of motor neuron disease (http://www.mndassociation.org). Other than life support management, no effective treatments exist for ALS (Zoccolella et al. 2009).

It is still not understood why specific neuronal populations are selectively vulnerable in ALS, such as certain somatic motor neurons and interneurons (Rowland and Shneider 2001; Sathasivam et al. 2001; Martin 2010a). The molecular pathogenesis of ALS is understood poorly, contributing to the lack of appropriate target identification and effective mechanism-based therapies to treat even the symptoms of this disease. At least two forms of ALS exist: idiopathic (sporadic) and heritable (familial). Most people with ALS have no known genetic contributions and are designated sporadic. Aging is a strong risk factor for ALS because the average age of onset is 55 (ALS Association, www.alsa.org). Familial forms of ALS 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 (Table 1) encoding SOD1 (*ALS1*), Alsin (*ALS2*), senataxin (*ALS4*), fused in sarcoma (FUS, *ALS6*), vesicle associated membrane protein (VAMP/synaptobrevin)-associated protein B (VAPB, *ALS8*), p150 dynactin (DCTN1), TAR-DNA binding protein (TADBP or TDP43, *ALS10*) and optineurin (*ALS12*) (Deng et al. 1993;
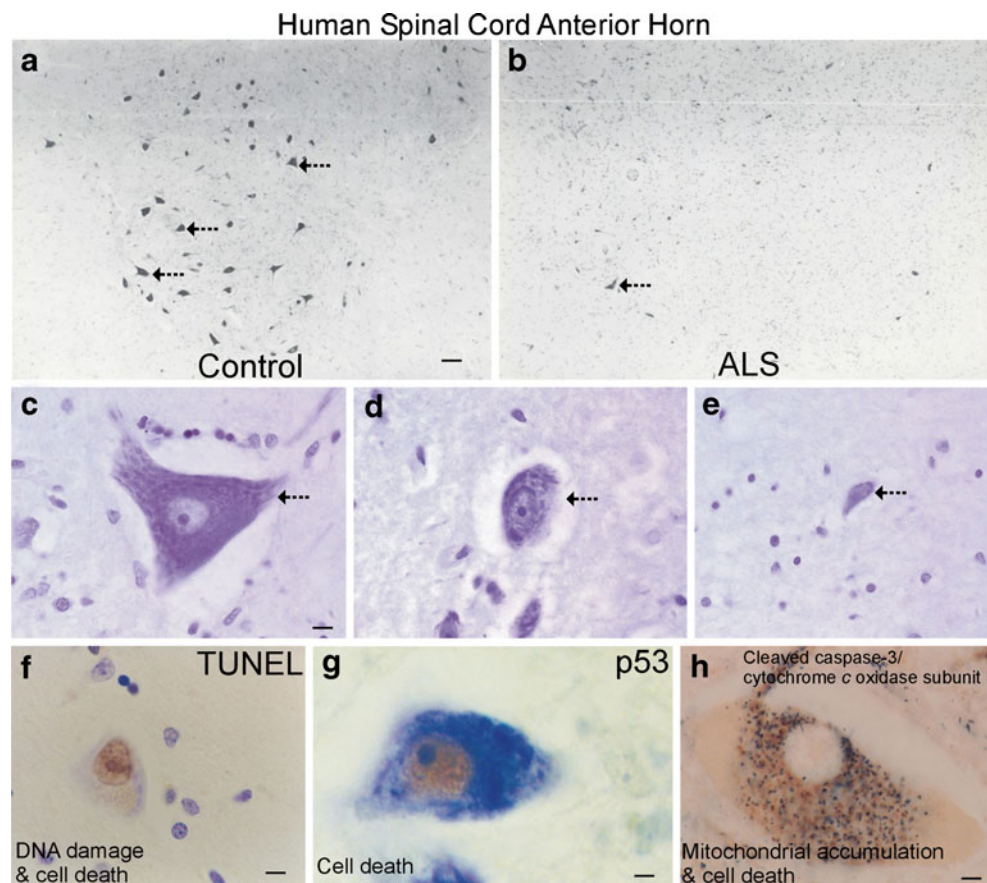
Human Spinal Cord Anterior Horn

Fig. 1 Motor neurons in spinal cord degenerate in people with ALS. **a** In normal control individuals, the anterior horns of the spinal cord contain many large, multipolar motor neurons (large dark cells, hatched arrows). **b** In ALS cases, the anterior horn is depleted of large neurons (dark cell, hatched arrow) and remaining neurons are atrophic. These attritional chromatolytic motor neurons display a dark condensed nucleus as seen microscopically. Scale bar in A=76 μm (same for b). **c**, **d**, and **e** Nissl staining shows that the degeneration of motor neurons in human familial ALS is characterized by shrinkage and progressive condensation of the cytoplasm and nucleus. The motor neuron in **c** (arrow) appears normal. It has a large, multipolar cell body and a large nucleus containing reticular network of chromatin and a large nucleolus. Scale bar=7 μm (same for d,e).The motor neuron in **d** (arrow) has undergone severe somatodentritic attrition. The motor neuron in **e** is at near endstage degeneration (arrow). The cell has shrunken to about 10% of normal size and has become highly condensed. The cell in **e** is identified as a residual motor neuron based the large nucleolus and residual large Nissl bodies. **f** Cell death assays (e.g., TUNEL) identify subsets of motor neurons in the process of DNA fragmentation. Nuclear DNA fragmentation (brown labeling) occurs in motor neurons in people with ALS as the nucleus condenses and the cell body shrinks. Motor neurons in the somatodendritic attrition stage (Martin 1999) accumulate DNA double strand breaks. Scale bar=7 μm. **g** In individuals with ALS, the tumor suppressor p53 accumulates in the nucleus (brown labeling) of motor neurons. Scale bar=5 μm, **h** Degenerating motor neurons in human ALS are immunopositive for cleaved caspase-3 (black-dark green labeling) in the somatodendritic attrition stage (Martin 1999). Motor neurons show prominent accumulation around the nucleus (pale circle) of discrete mitochondria (brown-orange labeling, detected with antibody to cytochrome *c* oxidase subunit I) exhibiting little light microscopic evidence for swelling. Scale bar=5 μm

Schymick et al. 2007; Kabashi et al. 2008; Vance et al. 2009; Maruyama et al. 2010). Variations in the phosphoinositide phosphatase *FIG4* gene cause *ALS11* (Chow et al. 2009). Several other genes are believed to be susceptibility factors for ALS (Table 1) (Saeed et al. 2006; Elden et al. 2010).

Evidence for mitochondrial dysfunction in human ALS

Human ALS is associated with mitochondrial abnormalities. Structural abnormalities in mitochondria are seen by electron microscopy (EM) in skeletal muscle, liver, spinal motor neurons and motor cortex of ALS patients (Sasaki and Iwata 1999; Menzies et al. 2002). A mutation in cytochrome *c* oxidase subunit I was found in a patient with a motor neuron disease phenotype (Comi et al. 1998). Another patient with motor neuron disease had a mutation in a mitochondrial tRNA gene (Borthwick et al. 2006). One type of mitochondrial DNA (mtDNA) mutation, called the common mtDNA deletion (mtDNA4977), is found non-uniformly within different human brain areas in aging; the highest levels are detected in the striatum and substantia nigra (Soong et al.

Table 1 Mutant/polymorphic genes linked to ALS

Locus	Inheritance	Gene	Protein Name & Function
ALS1/21q22	autosomal dominant (adult onset)	SOD1	Cu/Zn superoxide dismutase; dismutation of supeoxide
ALS2/2q33.2	autosomal recessive (juvenile onset primary lateral sclerosis)	Alsin	Alsin/guanine exchange factor for RAB5A and Rac1
ALS4/9q34	autosomal dominant (adult onset)	SETX	Senataxin/helicase; RNA processing
ALS6/16q12	autosomal recessive (adult onset)	FUS	Fused in sarcoma, component of heterogeneous nuclear ribonuclear protein complex; RNA/DNA binding protein
ALS8/20q13.33	autosomal dominant (adult onset)	VAPB	VAMP-associated protein B/part of SNARE complex
2q13	autosomal dominant (adult onset, atypical ALS)	DCTN1	Dynactin p150^{glued}/axonal transport, link between dynein and microtubule network
ALS10/1p36.22	autosomal dominant & sporadic (adult onset)	TARDBP	TAR DNA binding protein, DNA and RNA binding protein; regulates RNA splicing
ALS11/6q21	autosomal recessive (adult onset)	FIG4	FIG4 homolog, SAC1 lipid phosphatase domain containing; regulates phosphotidylinositol turnover
ALS12/10p13	autosomal recessive & dominant (adult onset)	OPTN	Optineurin; inhibits NFκB activation
14q11.1-q11.2	susceptibility factor	ANG	Angiogenin; angiogenesis; stimulates production of rRNA
22q12.2	susceptibility factor	NEFH	Neurofilament, heavy polypeptide; neurofilament subunit
12q12-q13	susceptibility factor	PRPH	Peripherin; intermediate filament formation
5q13	susceptibility factor	SMN1/SMN2	Survival motor neuron; RNA processing
7q36.6	susceptibility factor	DPP6	Dipeptidyl-peptidase 6; S9B serine protease, binds voltage-gated potassium channels
7q21.3	susceptibility factor for sporadic	PON1	Paraoxonase; organophasphate pesticide/nerve agent detoxification
12q24.1	susceptibility factor for sporadic	ATXN2	Ataxin-2; RNA processing

1992; Corral-Debrinski et al. 1992). However, no significant accumulation of the 5 kb common deletion in mtDNA has been found by single-cell analysis of motor neurons from sporadic ALS cases compared to controls (Mawrin et al. 2004). Overall, there is a lack of strong direct evidence for mitochondrial abnormalities participating in disease-causing mechanisms of human ALS, despite many associational/correlative data from human and animal/cell models.

Intracellular Ca^{2+} abnormalities and excitotoxicity are suspected links to mitochondrial dysfunction and oxidative stress in ALS (Beal 2002; Martin 2010a, b). Mitochondria regulate cytoplasmic Ca^{2+} levels (Babcock and Hille 1998; Nicholls 2002; Zorov et al. 2007). Electron microscopy on skeletal muscle biopsies of people with sporadic ALS shows changes indicative of elevated Ca^{2+} in motor neuron terminals, with some mitochondria showing an augmented Ca^{2+} signal (Siklos et al. 1996). Excitotoxicity has been implicated in the pathogenesis of ALS for a long time (Rothstein et al. 1992) and is another possible mechanism of motor neuron damage in ALS (Heath et al. 2002; Martin 2010a). Many sporadic ALS patients have reduced levels of synaptosomal high-affinity glutamate uptake (Rothstein et al. 1992) and astroglial glutamate transporter EAAT2 (excitatory amino acid transporter 2 or GLT1) in motor cortex and spinal cord (Rothstein et al. 1995). These changes could likely be secondary; however, reductions in levels of activity of EAAT2 in spinal cord might increase the extracellular concentrations of glutamate at synapses on motor neurons. Motor neurons might be particularly sensitive to glutamate excitotoxicity because they have a low proportion of GluR2-edited or under-edited AMPA subtype glutamate receptor on their surfaces, predisposing these cells to risk of excess Ca^{2+} entry and mitochondrial perturbations (Heath et al. 2002; Kwak and Kawahara 2005). Cell culture studies show that excess glutamate receptor activation in neurons can cause increased intracellular Ca^{2+}, enhanced mitochondrial reactive oxygen species (ROS) production, bioenergetic failure, and mitochondrial trafficking abnormalities (Chang and Reynolds 2006). Ca^{2+}-induced generation of ROS in brain mitochondria is mediated by mitochondrial permeability transition (Hansson et al. 2008). Motor neurons are particularly affected by inhibition of mitochondrial metabolism which can cause elevated cytosolic Ca^{2+} levels, excitability, and oxidative stress (Bergmann and Keller 2004). Recent data have provided a fresh prespective on ALS pathogenesis by demonstrating that motor neurons in transgenic (tg) ALS mice have faulty synaptic inhibition long before disease symptoms emerge, suggesting that inhibitory glycine receptor and interneuron abnormalities are upstream mechanisms while, possibly,

excitotoxicity is secondary (Chang and Martin 2009; Chang and Martin 2011). While many drugs targeting excitotoxicity as a mechanism have failed in human ALS clinical trials, the drug riluzole, a blocker of tetrodotoxin-sensitive sodium channels, is currently approved by the Food and Drug Administration for ALS treatment with marginal efficacy (Zoccolella et al. 2009).

Markers of oxidative stress and ROS damage are elevated in postmortem human ALS tissues (Beal 2002). In sporadic and familial ALS, protein carbonyls are elevated in motor cortex (Ferrante et al. 1997). Tyrosine nitration is elevated in human ALS nervous tissues (Abe et al. 1995; Beal et al. 1997; Sasaki et al. 2000). Studies of respiratory chain enzyme activities are discrepant. Experiments on autopsy tissue have shown increases in complex I, II, and III activities in vulnerable and non-vulnerable brain regions in patients with familial ALS-mutant SOD1 (Browne et al. 1998), but experiments by others show decreased complex IV activity in spinal cord ventral horn (Borthwick et al. 1999) and skeletal muscle (Vielhaber et al. 2000) of sporadic ALS cases. In sporadic ALS skeletal muscle, reductions in activity of respiratory chain complexes with subunits encoded by mtDNA are associated with reduced mtDNA content (Vielhaber et al. 2000) and decreased nitric oxide synthase (NOS) levels (Soraru et al. 2007). Alterations in skeletal muscle mitochondria are progressive (Echaniz-Laguna et al. 2006) and could be intrinsic to skeletal muscle and disease-causing, as suspected in human SOD1 tg mice (Wong and Martin 2010), rather than merely due to neurogenic atrophy, as assumed commonly.

Mitochondrial-orchestrated programmed cell death (PCD) involving p53 has been implicated in human ALS

PCD appears to contribute to the selective degeneration of motor neurons in human ALS, albeit seemingly as a non-classical form differing from apoptosis (Fig. 1) (Martin 1999; Martin and Liu 2004; Martin 2010a). Motor neurons appear to pass through sequential stages of chromatolysis, suggestive of initial axonal injury (Lieberman 1971), somatodendritic attrition without extensive cytoplasmic vacuolation, and then nuclear DNA fragmentation, nuclear and chromatin condensation, and cell death (Fig. 1) (Martin 1999). Motor neurons in people that have died from sporadic ALS and familial ALS show the same type of degeneration (Martin 1999). This cell death in human motor neurons is idefined 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 perikaryal mitochondria, cytochrome c, and cleaved caspase-3 (Fig. 1h) (Martin 1999; Martin and Liu 2004; Ginsberg et al. 2006). p53 protein also increases

in vulnerable CNS regions in people with ALS, and it accumulates specifically in the nucleus of lower and upper motor neurons with nuclear DNA damage (Fig. 1g) (Martin 2000; Martin et al. 2000). This p53 is active functionally because it is phosphorylated at serine-392 and has increased DNA binding activity (Martin et al. 2000). However, the morphology of this cell death is distinct from classical apoptosis, despite the nuclear condensation (Martin et al. 1998; Martin 1999; Martin 2010a). 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) (Martin 1999). In marked contrast, Bcl-2 protein is depleted severely in mitochondria-enriched fractions of affected regions and is sequestered in the cytosol (Martin 1999). Although these western blot results lacked direct specificity for motor neuron events (Martin 1999), subsequent immunohistochemistry (Martin and Liu 2004) and laser capture microdissection of motor neurons combined with mass spectroscopy-protein profiling (Ginsberg et al. 2006) have confirmed the presence of intact active caspase-3 in human ALS motor neurons.

Studies (Martin 1999; Martin 2000; Martin and Liu 2004) support the concept of an aberrant re-emergence of a mitochondrial-directed PCD mechanism, involving p53 activation and redistributions of mitochondrial cell death proteins, participating in the pathogenesis of motor neuron degeneration in human ALS (Fig. 1). The morphological and biochemical changes seen in human ALS are modeled robustly and faithfully at morphological and molecular levels in axonal injury/target deprivation (axotomy) models of motor neuron degeneration in adult rodents (Martin et al. 2000; Martin et al. 2005) but not in the current most commonly used human mutant SOD1 tg mouse models (Martin and Liu 2004; Martin et al. 2007). However, a new tg mouse expressing human mutant SOD1 only in skeletal muscle develops a motor neuron disease phenotype with morphological and biochemical changes very similar that those seen in human ALS motor neurons (Wong and Martin 2010).

Mitochondrial pathobiology in cell and mouse models of ALS

A common genetic mutation in human SOD1 that is linked to familial ALS (Table 1) is the substitution of glycine by alanine at position 93 (G93A) (Turner and Talbot 2008). SOD1 (also called Cu/Zn SOD) is a metalloenzyme of 153 amino acids (~16 kDa) that binds one copper ion and one zinc ion per subunit and is active as a ~32 kDa non-covalently linked homodimer (McCord and Fridovich 1969; Fridovich

1995). SOD1 is responsible for the detoxification and maintenance of intracellular $O_2^{\bullet-}$ concentration in the low femtomolar range by catalyzing its dismutation (McCord and Fridovich 1969; Fridovich 1995). SOD1 is ubiquitous (intracellular SOD concentrations are typically ~10-40 micromolar) in most tissues, possibly with highest levels in neurons (Rakhit et al. 2004).

Cell culture experiments reveal mitochondrial dysfunction in the presence of human mutant SOD1 (mSOD1) (Ferri et al. 2006). Expression of several mSOD1 variants increases mitochondrial superoxide levels and causes toxicity in rat primary embryonic motor neurons (Estévez et al. 1999), human neuroblastoma cells (Flanagan et al. 2002), and mouse neuroblastoma-spinal cord (NSC)-34 cells, a hybrid cell line with some motor neuron-like characteristics produced by fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells (Bilsland et al. 2008).These responses can be attenuated by over-expression of MnSOD (Flanagan et al. 2002). ALS-mSOD1 variants, compared to human 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 (Ferri et al. 2006).

Gurney and colleagues were the first to develop tg mice that express human mSOD1 (Gurney et al. 1994; Dal Canto and Gurney 1994; Chiu et al. 1995). Now, these mice are used widely as an animal model of ALS (Martin and Liu 2004; Turner and Talbot 2008). In the otiginal tg mice, human mSOD1 was expressed ubiquitously driven by its endogenous promoter in a tissue/cell non-selective pattern against a background of normal wildtype mouse SOD1 (Gurney et al. 1994). Effects of this human mutant gene in mice are profound. Hemizygous tg mice expressing high copy number of the G93A variant of human SOD1 become completely paralyzed and die at ~16–18 weeks of age (Gurney et al. 1994). G93A-mSOD1 mice with reduced transgene copy number have a much slower disease progression and die at ~8–9 months of age (Gurney et al. 1994; Martin et al. 2009).

Spinal motor neurons and interneurons in mice expressing G93A[high]-mSOD1 undergo prominent degeneration; about 60%–80% of lumbar motor neurons are eliminated by end-stage disease (Chiu et al. 1995; Mohajeri et al. 1998; Martin et al. 2007). Subsets of spinal interneurons degenerate before motor neurons in G93A[high]-mSOD1 (Martin et al. 2007; Chang and Martin 2009) and some are the glycinergic Renshaw cells (Chang and Martin 2009). Unlike the degeneration of motor neurons in human ALS (Martin 1999), motor neurons in G93A[high]-mSOD1 mice do not degenerate with a morphology resembling any form of apoptosis or apoptosis-necrosis hybrids (Martin 2010a). The motor neurons degeneration seen in G93A[high]-mSOD1 mice

more closely resembles a prolonged necrotic-like cell death process (Martin et al. 2007) involving early-occurring mitochondrial damage, cellular swelling, and dissolution (Kong and Xu 1998; Bendotti et al. 2001; Jaarsma et al. 2001; Sasaki et al. 2004). Biochemically, the death of motor neurons in G93A[high]-mSOD1 is characterized by cell body and mitochondrial swelling and formation of DNA single-strand breaks prior to double-strand breaks occurring in nuclear DNA and mtDNA (Martin et al. 2007). The motor neuron death in G93A[high]-mSOD1 mice is independent of activation of caspases-1 and −3, and also appears to be independent of capsase-8 and AIF activation within motor neurons (Martin et al. 2007). Indeed, caspase-dependent and p53-mediated apoptosis mechanisms might be blocked actively in G93A[high]-mSOD1 mouse motor neurons, possibly by up-regulation of inhibitors of apoptosis and changes in the nuclear import of proteins (Martin et al. 2007). More work is needed on the cell death and its mechanisms in G93A[low]-mSOD1 mice, because these mice could be more relevant physiologically and preclinically to the human disease compared to G93A[high]-mSOD1 mouse.

Mitochondrial disease has been implicated in the mechanisms of motor neuron degeneration in tg mSOD1 mouse models, but until recently, most evidence has been circumstantial. In different mSOD1 mouse models of ALS, mitochondria in spinal cord neurons exhibit prominebt structural pathology (Wong et al. 1995; Kong and Xu 1998; Bendotti et al. 2001; Jaarsma et al. 2001; Sasaki et al. 2004; Martin et al. 2007), and some of the mitochondrial degeneration occurs very early in the course of the disease (Bendotti et al. 2001; Martin et al. 2007). Mitochondrial microvacuolar damage in motor neurons is seen by electron microscopy at 4 weeks of age in G93A[high] mice (Martin et al. 2007). It has been argued that mitochondrial damage in G93A[high]-mSOD1 mice is related to supra-normal levels of SOD1 and might not be related causally to the disease process because tg mice expressing high levels of human wild-type SOD1 show some mitochondrial pathology (Jaarsma et al. 2001), but mitochondrial abnormalities in motor neurons have been found histologically also in G93A[low]-mSOD1 mice (Sasaki et al. 2004) and in mice with only skeletal muscle expression of human SOD1 (Wong and Martin 2010). Thus, mitochondria could be primary sites of human SOD1 toxicity in tg mice irrespective of transgene copy number, tissue expression, and expression level of human SOD1, but direct, unequivocal causal relationships have been lacking.

Mutated and wild-type forms of human SOD1 can cause ALS in tg mice (Wong and Martin 2010). Human mSOD1 proteins appear to acquire a toxic property or function, rather than having diminished O_2^- scavenging activity (Deng et al. 1993; Borchelt et al. 1994; Yim et al. 1996). Wild-type SOD1 can gain toxic properties through loss of Zn (Estévez et al. 1999) and oxidative modification (Kabashi et al. 2007;

Ezzi et al. 2007). A gain in aberrant oxidative chemistry appears to contribute to the mechanisms of mitochondriopathy in G93A[high] mice (Beckman et al. 1993; Liochev and Fridovich 2003). G93A-mSOD1 has enhanced free radical-generating capacity compared to wild-type enzyme (Yim et al. 1996) and can catalyze protein oxidation by hydroxyl-like intermediates and carbonate radical (Pacher et al. 2007). G93A[high] mice have increased protein carbonyl formation in total spinal cord tissue extracts at pre-symptomatic disease (Andrus et al. 1998). Protein carbonyl formation in mitochondrial membrane-enriched fractions of spinal cord is a robust signature of incipient disease (Andrus et al. 1998). A mass spectroscopy study of G93A[high] mice identified proteins in total spinal cord tissue extracts with greater than baseline carbonyl modification, including SOD1, translationally controlled tumor protein, and UCHL1 (Poon et al. 2005). Nitrated and aggregated cytochrome c oxidase subunit-I accumulate in G93A[high] mouse spinal cord (Martin et al. 2007). Nitrated MnSOD accumulates also in G93A[high] mouse spinal cord (Martin et al. 2007). Toxic properties of mSOD1 might also be mediated through protein binding or aggregation. Endogenous mouse SOD1 (Okado-Matsumoto and Fridovich 2001) and human wild-type SOD1 and mSOD1 (Higgins et al. 2002) associate with mitochondria. Human SOD1 mutants associate with spinal cord mitochondria in mSOD1 mice and can bind Bcl-2 (Pasinelli et al. 2004), thus potentially being decoys or dominant negative regulators of cell survival molecules, but it is not known if this process is occurring specifically in motor neurons. This observation is intriguing considering the finding that mitochondrial Bcl-2 is depleted in human ALS (Martin 1999). Binding of mSOD1 (and perhaps its low-mobility species) to mitochondria has been reported to be spinal cord selective and age-dependent (Pasinelli et al. 2004), but this work also 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 (Goldsteins et al. 2008). Human SOD1 mutants can also shift mitochondrial redox potential when expressed in cultured cells (Ferri et al. 2006). Nevertheless, the direct links between the physicochemical changes in wild-type and mutant SOD1 and the mitochondrial functional and structural changes associated with ALS and motor neuron degeneration remain uncertain.

EM studies of motor neurons in G93A[high] mice have shown that the outer mitochondrial membrane (OMM) remains relatively intact to permit formation of mega-mitochondria (Kong and Xu 1998; Martin et al. 2007; Martin et al. 2009). 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 motor neuron cell bodies show major structural changes (Martin et al. 2009). Thus, disease might start distally in mitochondria of motor neuron processes (Martin et al. 2007; Martin et al. 2009). Another interpretation of ultrastructural findings is that the mSOD1 causes mitochondrial degeneration by inducing OMM extension and leakage and intermembrane space expansion (Higgins et al. 2003). Mechanisms for this damage could be related to mSOD1 gaining access to the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich 2001; Higgins et al. 2002; Higgins et al. 2003). This mitochondrial conformation seen by EM might favor the formation of the mitochondrial permeability transition pore (Crompton 2004; Bernardi et al. 2006; Zorov et al. 2007); indeed, we found evidence for increased contact sites between the OMM and IMM in dendritic mitochondria in G93A[high] mice (Martin et al. 2009). Another feature of motor neurons in young G93A[high] mice, before symptoms emerge, is apparent fission of ultrastructurally normal mitochondria in cell bodies and fragmentation of abnormal mitochondria (Martin et al. 2009). 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 could be important to the pathobiology of mSOD1 toxicity to motor neurons in G93A[high] mice.

We have hypothesized that mitochondrial trafficking perturbations occur in motor neurons of mSOD1 (Martin et al. 2007). Some data support the novel idea that mitochondria might act as messengers from distal regions (axon branches and dendrites) of motor neurons in mSOD1 mice. G93A[high]-mSOD1 mouse motor neurons accumulate mitochondria from the axon terminals and generate higher levels of superoxide, nitric oxide (NO), and peroxynitrite (ONOO[-]) than motor neurons in tg mice expressing human wild-type SOD1 (Martin et al. 2007). This mitochondrial accumulation occurs at a time when motor neuron cell body volume is increasing, suggestive of ongoing abnormalities with ATP production or plasma membrane Na^+,K^+ ATPase (Martin et al. 2007). G93A-mSOD1 perturbs anterograde axonal transport of mitochondria in cultured primary embryonic motor neurons (De Vos et al. 2007) making it possible that retrogradely transported mitochondria with toxic properties from the neuromuscular junction fail to be returned to distal processes (Martin et al. 2007). Mitochondria with enhanced toxic potential from distal axons and terminals could therefore have a "Trojan horse" role in triggering degeneration of motor neurons in ALS via retrograde transport from diseased skeletal muscle.

Motor neurons in G93A[high]-mSOD1 mice also accumulate higher levels of intracellular Ca^{2+} than motor neurons in age-match control mice (Martin et al. 2007). This finding could be relevant to activation of the mitochondrial permeability transition pore (Zoratti and Szabo 1995; Crompton 1999; Leung and halestrap 2008). The intracellular Ca^{2+} signal in motor neurons is very compartmental and mitochondrial-like in its appearance (Martin et al. 2005; Martin et al. 2007). Abnormal elevations of intracellular Ca^{2+} in G93A[high]-mSOD1 mouse motor neurons have been seen also by different Ca^{2+} detection methods (Siklos et al. 1998; Jaiswal and Keller 2009). Recent work on a mouse neuromuscular junction preparation has shown that mitochondrial Ca^{2+} accumulation is accompanied by greater mitochondrial depolarization, specifically within motor neuron terminals of human mutant SOD1 tg mice (Nguyen et al. 2009).

NO signaling mechanisms in mitochondria of ALS mice appear to be involved in the pathogenesis. Motor neurons seem to be unique regarding NO production because they express constitutively low levels of inducible NO synthase (iNOS) (Martin et al. 2005; Martin et al. 2007; Chen et al. 2010). G93A[high]-mSOD1 mouse motor neurons accumulate nicotinamide adenine dinucleotide phosphate diaphorase and iNOS-like immunoreactivity (Martin et al. 2007; Chen et al. 2010). iNOS is also up-regulated aberrantly in human sporadic ALS motor neurons (Sasaki et al. 2000). *iNos (Nos2)* gene knockout (Martin et al. 2007) and iNOS inhibition with 1,400 W (Chen et al. 2010) extend significantly the lifespan of G93A[high]-mSOD1 mice. Thus, mitochondrial oxidative stress, Ca^{2+} dysregulation, iNOS activation, protein nitration, and protein aggregation (not necessarily SOD1 aggregation) are all likely intrinsic, cell-autonomous mechanisms in the process of motor neuron degeneration caused by mSOD1 in mice (Chen et al. 2010). The mechanistic basis for the differences between human ALS and mSOD1 mice, regarding cell death phenotype (Martin 2010a; Martin 2010b) is not yet clear, but could be related to the extreme non-physiological expression of toxic mSOD1 or to fundamental differences in cellular death mechanisms in human and mouse neurons or tissue inflammation that drive motor neurons in mSOD1 tg mice to necrotic-like death according to the cell death matrix concept (Martin 2010a). Another contributing factor for this difference between human and mouse motor neurons is that mitochondria are functionally diverse and have species-specific activities and molecular compositions, including the makeup of the mitochondrial permeability transition pore (Kunz 2003). These possibilities allow for skepticism regarding the suitability of previous tg mSOD1 mouse lines to model human ALS. Therefore, we have recently created a tg mouse with restricted expression of human SOD1 in skeletal muscle that develop ALS with a motor neuron degeneration phenotype similar to that seen in human ALS (Wong and Martin 2010).

The mPTP contributes to disease mechanisms in ALS 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 lead to new mechanism-based treatments for ALS. One specific therapeutic target of investigation for mitochondria in disease causality in ALS is the mPTP (Martin 2010c).

The mPTP was first implicated in mouse ALS pathogenesis using pharmacological approaches. Cyclopsorine A treatment of G93A[high] mice, delivered into the cerebral ventricle or systemically to mice on a multiple drug resistance type 1a/b background (to inactivate the blood-brain barrier), improved outcome modestly (Keep et al. 2001; Karlsson et al. 2004; Kirkinezos et al. 2004). These studies were confounded by the immunosuppressant actions of cyclopsorine A through calcineurin inhibition. Pharmacological studies using cyclophilin D (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 the voltage-dependent anion channel (VDAC) and the 18 kDa translocator protein (TSPO, or peripheral benzodiazepine receptor), improved motor performance, delayed disease onset, and extended survival of G93A[high] mice (Bordet et al. 2007). However, another study using a different TSPO ligand (Ro-4864) did not show positive effects with G93A[high] mice (Mills et al. 2008).

We identified CyPD and the adenine nucleotide translocator (ANT) as targets of nitration in ALS mice (Martin et al. 2009). CyPD nitration is elevated in early- to mid-symptomatic stages, but declines to baseline at end-stage disease (Martin et al. 2009). ANT nitration is pertinent because it occurs in pre-symptomatic and symptomatic stages but not at end-stage disease or in tg mice expressing human wild-type SOD1 (Martin et al. 2009). The ANT is important in the context of age-related neurodegenerative disease because it undergoes carbonyl modification during aging as seen in housefly flight muscle (Yan and Sohal 1998) and rat brain (Prokai et al. 2007). *In vitro* cell-free and cell experiments have shown that NO and $ONOO^-$ can act directly on the ANT to induce mitochondrial permeabilization in a cyclosporine A-sensitive manner (Vieira et al. 2001). Oxidative stress enhances the binding of CyPD to ANT (McStay et al. 2002). Some SOD1 mutants are unstable and lose copper (Trumbull and Beckman 2009), and interestingly, copper interactions with ANT and thiol modification of ANT can cause mPTP opening (Costantini et al. 2000; García et al. 2007). Together these data and future work could reveal that oxidative and nitrative damage to proteins, some of which are components of the mPTP

(Bernardi et al. 2006: Leung and Halestrap 2008), in G93A[high] mice is targeted rather than stochastic and could impinge on the functioning of the mPTP.

We examined directly the role of CyPD in the process of motor neuron disease in ALS mice through gene-ablation (Martin et al. 2009). G93A[high]-mSOD1 mice without CyPD show markedly delayed disease onset and lived significantly longer than tg mice with CyPD. The effect of CyPD deletion was much more prominent in female mice than in male mice (Martin et al. 2009). Female mice showed positive effects with only haplo-deletion of CyPD. *Ppif* gene ablation in tg mice with much lower levels of human mSOD1 expression and a slower disease progression (G93A[low]-mSOD1 mice) also show significantly delayed disease onset and lived significantly longer than tg mice with CyPD (Martin et al. 2009). Thus, some form of mPTP pathobiology is occurring regardless of whether transgene expression of G93A is high or low.

Nevertheless, most G93A-mSOD1 mice without CyPD develop eventually motor neuron disease and die. Other work on CypD null mice has shown that high concentrations of Ca^{2+} (2 mM) can still lead to mPTP activation without CyPD and that cell deaths caused by Bid, Bax, DNA damage and TNF-α still occur without CyPD (Grimm and Brdiczka 2007). The effects of CyPD deficiency on motor neuron cell death mechanisms thus need detailed examination, but the cell death phenotype might switch or convert to another form with the attenuation of mitochondrial swelling. A switch in the cell death morphology and molecular mechanisms in motor neurons of mSOD1 mice without CyPD is an outcome consistent with the cell death matrix concept (Martin 2010a).

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