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REVIEW

Mitochondrial dysfunction in amyotrophic lateral sclerosis – a valid pharmacological target?

H Muyderman and T Chen

Centre for Neuroscience and *Discipline of Medical Biochemistry, Flinders Medical Science and Technology, School of Medicine, Flinders University, Adelaide, SA, Australia*

Correspondence
Dr Hakan Muyderman, Discipline of Medical Biochemistry, School of Medicine, Flinders University, GPO Box 2100, Adelaide, SA 5041, Australia. E-mail: hakan.muyderman@flinders.edu.au

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the selective death of upper and lower motor neurons which ultimately leads to paralysis and ultimately death. Pathological changes in ALS are closely associated with pronounced and progressive changes in mitochondrial morphology, bioenergetics and calcium homeostasis. Converging evidence suggests that impaired mitochondrial function could be pivotal in the rapid neurodegeneration of this condition. In this review, we provide an update of recent advances in understanding mitochondrial biology in the pathogenesis of ALS and highlight the therapeutic value of pharmacologically targeting mitochondrial biology to slow disease progression.

LINKED ARTICLES
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Abbreviations
AIF, apoptosis-inducing factor; ALS, amyotrophic lateral sclerosis; ETC, electron transport chain; fALS, familial ALS; PGC-1α, PPARγ co-activator 1α; PPX, pramipexole; SOD1, superoxide dismutase

Introduction

Amyotrophic lateral sclerosis (ALS) is a lethal disorder of incompletely understood aetiology encompassing a range of clinicopathological entities (Turner *et al.*, 2013). The disease is characterized by selective death of upper motor neurons in the cerebral cortex and of lower motor neurons in the brainstem and spinal cord resulting in paralysis, disability and death within 1 to 5 years from diagnosis. Moreover, there is no cure or effective treatment. Most cases of ALS are sporadic but about 5% are familial (fALS) and 20% of these are caused by mutations in the gene for superoxide dismutase (SOD1) (Robberecht and Philips, 2013). Recently, a number of new genes have been causally linked to the pathogenesis of fALS. These include genes directly linked to RNA metabolism such as TARDBP that encodes for the TAR DNA binding protein TDP-43 (Sreedharan *et al.*, 2008) and the Fused-in-Sarcoma (FUS) encoding gene FUS/TLD (Vance *et al.*, 2009). fALS can also be caused by mutations in genes associated with protein degradation pathways, including ubiquilin-2 (UBQLN2) (Deng *et al.*, 2011), valosin-containing protein (VCP) (Johnson *et al.*, 2010) and the vesicle-associated membrane protein-associated protein B (VAPB) (Nishimura *et al.*, 2004), p62/sequestosome (SQSTM1) (Fecto *et al.*, 2011). More recently, hexanucleotide expansion in the C9ORF72 gene has been found to be a common genetic cause for ALS (Dejesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). The discovery of new disease-causing genes represents a paradigm shift in the understanding of the pathogenesis of ALS. At present, it is generally believed that dysfunctional RNA metabolism and the subsequent disturbance of protein homeostasis is fundamental to the pathogenesis of this condition.

The histopathological hallmark of ALS is the presence of cytosolic protein aggregates (Lansbury and Lashuel, 2006). Current evidence suggests that these protein aggregates interfere with normal cellular processes, resulting in oxidative stress, excitotoxicity, mitochondrial dysfunction and finally retraction of axons, synaptic disintegration and cell death. These observations have led to the postulation of the *'dying back'* hypothesis of motor neuron death in ALS (Redler and Dokholyan, 2012). There is also considerable evidence suggesting that the activation of caspase-dependent apoptotic pathways is involved in neurodegeneration in ALS

(Muyderman *et al.*, 2009; Martin, 2011; Soo *et al.*, 2012). However, whether caspase-activation represents the end point of other pathological processes or is a key process in ALS remains controversial and pharmacological approaches aimed at inhibiting apoptosis have yielded little or no success.

Mitochondrial dysfunction in ALS

Mitochondrial dysfunction has been implicated as playing a role in motor neuron death in ALS. Fragmentation of mitochondria and changes in mitochondrial morphology and expression of fusion/fission proteins are well described in ALS and have pronounced effects on normal mitochondrial function (Sasaki *et al.*, 2007). Defective mitochondrial transport may be responsible for the accumulation of abnormal mitochondria in motor neuron axons seen in animal models of ALS and also in human patients. Studies in cell culture models and in transgenic animals have demonstrated aberrations in oxidative metabolism linked to changes in electron transport chain (ETC) activity and impaired ATP production (Keep *et al.*, 2001; Mattiazzi *et al.*, 2002; Menzies *et al.*, 2002; Knott *et al.*, 2008). Mitochondria from ALS patients have impaired Ca^{2+} homeostasis and an increased production of reactive oxygen species (ROS) which is associated with oxidative-related damage including changes in protein carbonylates and tyrosine nitration (Beal *et al.*, 1997; Beal, 2002). Indeed, glutamate-receptor mediated neurotoxicity has been linked to an overload of mitochondrial calcium and ROS production in cultured spinal motor neurons from transgenic ALS animals (Carriedo *et al.*, 2000). Together, these studies demonstrate that changes in mitochondrial function and dynamics are a central and common feature of the pathogenesis in ALS. However, it is not known if mitochondrial dysfunction is a primary or secondary event in these processes.

Mitochondria-linked cell death pathways

Apoptotic cell death is intimately linked to mitochondrial dysfunction and commonly involves activation of caspases via the 'intrinsic' or the 'extrinsic' pathway (Figure 1) (Kroemer *et al.*, 1997; Kroemer, 2010; Sims and Muyderman, 2010). The intrinsic pathway is dependent on the mitochondrial release of proteins leading to the activation of caspases, particularly caspase-3, thereby initiating the apoptotic cascade that finally results in chromatin condensation and DNA fragmentation. The extrinsic pathway is initiated by the binding of specific ligands to plasma membrane cell

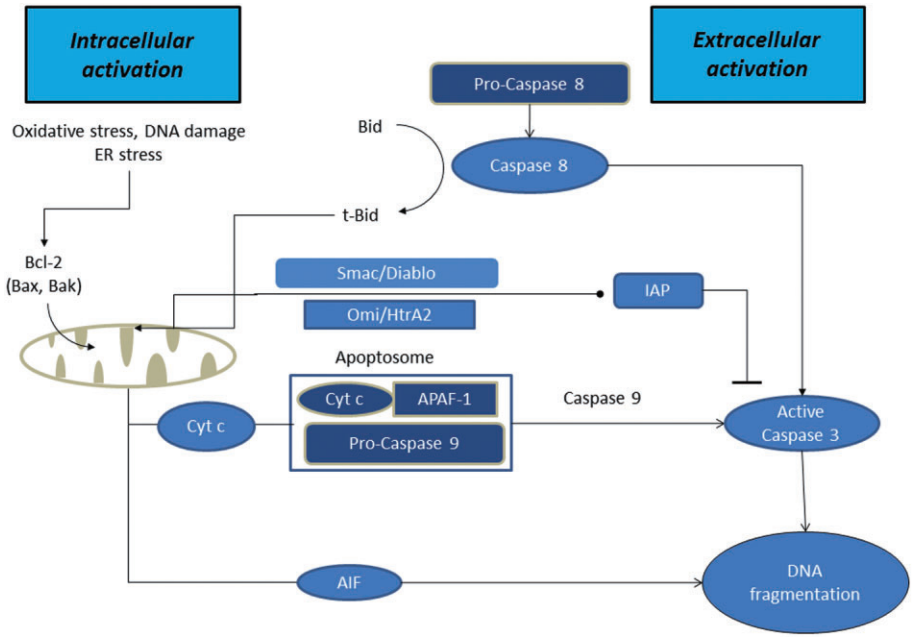

Figure 1

Proteins released from the intermembrane space and their contribution to apoptosis. Release of cytochrome c is a key step in the intrinsic pathway of apoptosis forming a complex known as an apoptosome with the proteins APAF-1 and procaspase-9 and with ATP. Apoptosome formation leads to activation of executioner caspases, particularly caspase-3 resulting in internuclear protein and DNA degradation. The process can be promoted by the release of Smac/DIABLO and/or Omi/HtrA2 that block the IAPs family of proteins that are endogenous inhibitors of caspases. Release of AIF may lead directly to a caspase-independent form of apoptosis after its interaction with cyclophilin A. The extrinsic pathway is initiated by the binding of specific ligands to plasma membrane cell death receptors such as FAS and DR6 triggering activation of caspase-8 which in turn activates other executioner caspases. Executioner caspase activation in the extrinsic pathway can occur without involvement of mitochondria but may also result in caspase-8 mediated cleavage of Bid to produce truncated Bid, promoting release of mitochondrial apoptogenic proteins producing a mixed intrinsic/extrinsic presentation.

death receptors such as FAS and DR6 , thereby triggering activation of caspase-8 which in turn activates other 'executioner' caspases (Kantari and Walczak, 2011). Executioner caspase activation in the extrinsic pathway can occur without the involvement of mitochondria (Galluzzi *et al.*, 2009; 2012) but may also result in caspase-8 -mediated cleavage of Bid to produce truncated Bid, thereby promoting the release of mitochondrial apoptogenic proteins and producing a mixed intrinsic/extrinsic response. In addition, mitochondria-mediated caspase-independent modes of apoptosis have been reported in neurodegenerative diseases involving release of the apoptosis-inducing factor AIF (Jordan *et al.*, 2003; Polster, 2013).

Mitochondrial release of large apoptogenic proteins, including cytochrome c, into the cytosol during the development of apoptosis requires a substantial increase in permeability of the outer mitochondrial membrane. Mechanisms underlying protein release from mitochondria are believed to involve the proapoptotic Bcl-2 family of proteins (Galluzzi *et al.*, 2009; 2012; Michels *et al.*, 2013). Although not fully understood, this process seems to involve the translocation of the two proapoptotic proteins Bim and Bax from the cytosol to the mitochondrial membrane, resulting in an increased permeability of the outer membrane. Movement of another Bcl-2 family protein, Bad, from the cytosol to the mitochondria can also contribute to membrane permeabilization under some circumstances and coupled with the activation of caspase-8 via the extrinsic apoptotic pathway can result in the cleavage of yet another Bcl-2 family protein, Bid, thereby promoting the release of proapoptotic proteins. Other members of the Bcl-2 family are anti-apoptotic. For example, the Bcl-2 homolog BCL-XL binds to BAX and BAK, preventing formation of pores in the outer membrane and hence limiting the release of apoptogenic proteins (Michels *et al.*, 2013). An alternative process concerns the formation of the mitochondrial transition pore which leads to permeabilization of the inner membrane. Subsequent swelling of the mitochondria due to water entry into the matrix following pore opening results in disruption of the outer membrane and the release of proteins from the intermembrane space (Brenner and Moulin, 2012). The opening of the transition pore is usually induced by abnormal accumulation of calcium but can be promoted by multiple factors including oxidative stress. The composition of the permeability transition pore is not fully understood but is believed to include an adenine nucleotide translocase in the inner mitochondrial membrane, a voltage-dependent anion carrier in the outer membrane and the matrix protein cyclophilin D (Brenner and Moulin, 2012).

Mutant SOD1, ALS and mitochondria

Significant advances in understanding the mechanisms of motor neuron pathology in ALS have come from studies using transgenic rodents expressing mutant forms of the human SOD1 (mutant SOD1) gene that mimic the familial form of ALS (Gurney *et al.*, 1994; Nagai *et al.*, 2001). At present there are more than 150 known mutations in the SOD1 gene. Animals expressing mutant SOD1 typically display a phenotype that resembles ALS and demonstrate most of the histopathological and biochemical features and also the symptoms of the human disease. Pathology first appears in motor neurons of the spinal cord within 6 weeks of birth and the first motor symptoms appear at 3 months of age resulting in a progressive paralysis similar to that found in humans (Chiu *et al.*, 1995; Julien and Kriz, 2006). Several studies have demonstrated a role for mutant SOD1 in mitochondrial dysfunction in ALS pathogenesis (see Table 1). Mutant SOD1 is often found as aggregates at the outer membrane of mitochondria in motor neurons of various mouse models and in fALS patients (Higgins *et al.*, 2003). Thus, it is believed that disruption of mitochondrial function by the presence of misfolded protein aggregates results in mitochondrial damage, including increased mitochondrial volume and excess superoxide production (Pasinelli *et al.*, 2004; Pickles *et al.*, 2013). Moreover, cells expressing some forms of mutant SOD1 undergo mitochondrial apoptotic signalling (see Figure 2: Table 1) and mutant SOD1 transgenic animals overexpress proapoptotic proteins such as the BH3-only protein Bim and Bax while Bcl-2 and Bcl-Xl have been found to be decreased (Vukosavic *et al.*, 1999; 2000). Overexpressing Bcl-2 delays caspase activation in the mutant $SOD1^{G93A}$ transgenic animal (Vukosavic *et al.*, 2000) and silencing Bim protein expression delays disease onset in other animal models of the disease. A similar anti-apoptotic effect is seen in cell culture models of mutant SOD1 ALS (Hetz *et al.*, 2007; Soo *et al.*, 2012). In mutant $SOD1^{G85R}$ -expressing Neuro2a cells, Bim deletion leads to reduced Bax recruitment to mitochondria and decreased cytochrome c redistribution. As Bim is considered a direct link between endoplasmic reticulum (ER) stress and mitochondrial apoptosis, these studies indicate a clear pathway to cell death mediated by mutant SOD1 involving ER stress (Soo *et al.*, 2012). Mutant SOD1 could also damage mitochondria directly (Figure 2). Mitochondria containing mutant $SOD1^{G93A}$, but not wild-type SOD1, display changes in volume, aggregation, fragmentation and vacuolization (Higgins *et al.*, 2003; Sasaki *et al.*, 2004). Moreover, findings in human post-mortem or biopsy samples have reported abnormal mitochondria in cell bodies of motor neurons, proximal axons and in intramuscular nerves and skeletal muscle (Chung and Suh, 2002; Echaniz-Laguna *et al.*, 2002; Sasaki and Iwata, 2007). Interestingly, such changes often precede disease onset in the mutant $SOD1^{G93A}$ transgenic mouse and occurs before any other signs of motor neuron degeneration (Kong and Xu, 1998). These early changes are often followed by a substantial increase in mitochondrial vacuolization at the time of symptom onset (Kong and Xu, 1998; Bendotti *et al.*, 2001). Other studies have shown that mutant SOD^{G37R} binds directly to a voltage-dependent anion channel in the outer mitochondrial membrane and that this interaction inhibits channel conductance (Israelson *et al.*, 2010). Moreover, deletion of this channel results in decreased lifespan of the G37R mouse. Together these and other studies strongly suggest a direct link between mitochondrial viability and motor neuron degeneration and are further supported by other studies demonstrating sequential activation of caspase-1 and 3, with caspase-1 activation occurring before the onset of symptoms and caspase-3 activation being associated with later motor neuron loss (see Table 1) (Pasinelli *et al.*, 2000). Consistent with these findings, treatment with a

Table 1

Mitochondrial dysfunction in ALS

Mitochondria dysfunctions	Model	Gene mutation	Major findings	References
Morphology, fusion/fission and transport	Mouse primary motor neurons	FUS R521G R521H	Shortened mitochondria in motor neurons expressing mutant FUS.	Sasaki *et al.*, 2007
	Tg rats	FUS R521C	Transgenic rats expressing mutant FUS showed ubiquitinated aggregates positive for the mitochondria marker COXIV	Huang *et al.*, 2011
	SH-SY5Y, NSC-34	SOD1 G93A	Mutant SOD1 increased mitochondria Opa1, decreased Drp1	Ferri *et al.*, 2010
	Tg mice	SOD1 G93A	Fusion proteins (Mfn1 and Opa1) and fission proteins (Drp1 and Fis1) increased.	Liu *et al.*, 2013
	Tg mice	SOD1 G93A	Reduced density of mitochondria in motor axons and motor neurons in the triangularis sterni	Marinkovic *et al.*, 2012
	Tg mice	SOD1 G93A	Decreased mitochondrial length and accumulation of fragmented mitochondria. Arrest in both anterograde and retrograde axonal transport and increased cell death	Song *et al.*, 2013
	Knockout mice	$p62^{-/-}$ (SQSTM1)	Fragmented mitochondria in $p62^{-/-}$ mouse embryonic fibroblast cells.	Lamar Seibenhener *et al.*, 2013
	Tg mice	wt-hTDP-43	Accumulation of mitochondria in TDP-43-negative cytoplasmic inclusions in motor neurons, lack of mitochondria in motor axon terminals.	Shan *et al.*, 2010
	Tg mice	wt-hTDP-43	Abnormal juxtanuclear aggregates of mitochondria accompanied by enhanced levels of Fis1 and phosphorylated DLP1; reduction in Mfn 1 expression	Xu *et al.*, 2011
	NSC-34	TDP-43 Q331K, M337V	Swollen mitochondria	Lu *et al.*, 2012
	Rat motor neurons Tg mice	Wt-hTD-P43 Q331K M337V	Overexpression of Wt and mutant TDP-43 resulted in reduced mitochondrial length and in neurites of primary motor neuron, suppression of TDP-43 resulted in significantly increased mitochondrial length and density in neuritis. Abnormal localization of TDP-43 in cytoplasm induced substantial and widespread abnormal mitochondrial dynamics. Co-expression of mitochondrial fusion protein mitofusin 2 (Mfn2) abolished TDP-43-induced mitochondrial dynamics abnormalities and mitochondrial dysfunction.	Wang *et al.*, 2013
	NSC-34	Wt-hTDP-43 Q331K M337V C-terminal fragment	Full length TDP-43 and its C-terminal fragment induced clustered and unevenly distributed mitochondria.	Hong *et al.*, 2012
	Human spine cord samples	NA	Accumulation of mitochondria in the somata, dendrites and proximal axons.	Sasaki *et al.*, 2007
	Rat cortical neurons	VAPB P56S	$VAPB^{P56S}$ selectively disrupted anterograde axonal transport of mitochondria, disrupted Ca^{2+} homeostasis and effected the Miro1/kinesin-1 interaction with tubulin.	Morotz *et al.*, 2012
	Knock-in mice	VCP R155H	Electron microscopic analysis of 19-month-old VCP^{R155H} knock-in mouse muscle exhibited extensive accumulation of abnormal mitochondria and vacuoles in the inter-myofibrillar space.	Nalbandian *et al.*, 2013

Table 1

Continued

Mitochondria dysfunctions	Model	Gene mutation	Major findings	References
Complex activity, oxidative phosphorylation and redox	NSC-34	SOD1	Presence of mutSOD1s resulted in impairment of the respiratory chain and a shift in the mitochondrial redox balance.	Ferri *et al.*, 2006
	Tg rats	SOD1 G93A	Mutant SOD1 up-regulated in the IMS, increased ROS production from the spinal cord at the presymptomatic stage	Ahtoniemi *et al.*, 2008
	Tg mice	SOD1 G93A	Deficiency in mitochondrial respiration, electron transfer chain, and ATP synthesis.	Mattiazzi *et al.*, 2002
	SH-SY5Y	SOD1 G93A, H80R	Impaired ATP production	Pesaresi *et al.*, 2011
	Tg mice	SOD1-G93A/ PGC-1α	PGC-1α restores mitochondrial electron transport chain activities in the spinal cord in double transgenic (SOD1-G93A/PGC1-α) mice.	Zhao *et al.*, 2011
	Tg mice	SOD1 G93A	Mitochondrial ETC activities are decreased in the ventral horn prior to the disease onset and during the course of disease progression	Jung *et al.*, 2002
	Tg rats	SOD1 G93A	Defective respiratory function in astrocytes: decreased oxygen consumption, lack of ADP-dependent respiratory control and decreased membrane potential.	Cassina *et al.*, 2008
	Knockout mice	$p62^{-/-}$ (SQSTM1)	Mitochondria in $p62^{-/-}$ cells displayed decreased mitochondrial membrane potential. Impairment in ATP production.	Lamar Seibenhener *et al.*, 2013
	SH-SY5Y	endogenous TDP-43	Paraquat, an inhibitor of the mitochondrial electron transport chain and inducer of superoxide/peroxynitrite stress, induced cytoplasmic accumulation of TDP-43 including aggregates of TDP-43 resembling RNA stress granules.	Meyerowitz *et al.*, 2011
	NSC-34	Wt-hTDP-43 Q331K M337V	Wt-TDP-43 as well as mutant impairs ATP generation.	Hong *et al.*, 2012
	SH-SY5Y Human primary fibroblasts, neurons and astrocytes Mice primary cortical neurons	VCP R155C R155H R191Q siRNA shRNA	VCP deficiency is associated with decreased mitochondrial membrane potential, ATP levels and increased mitochondrial respiration and oxygen consumption.	Bartolome *et al.*, 2013
	Human muscle samples		Cytochrome c oxidase deficiency.	Crugnola *et al.*, 2010
Calcium buffering	Tg mice	SOD1 G93A, G85R	Decreased mitochondrial Ca^{2+} loading capacity in brain and spinal cord prior to onset of motor symptoms.	Damiano *et al.*, 2006
	Tg mice	SOD1 G93A G37R, G85R	Deletion of cyclophilin D results in a chronic increase in mitochondrial buffering of Ca^{2+} and is associated with improved mitochondrial ATP synthesis, reduced mitochondrial swelling, and retention of normal morphology.	Parone *et al.*, 2013
	Tg mice hSOD1	SOD1 G93A/chat-GluR2	Cholinergic neuron-specific GluR2 overexpression resulted in reduction of Ca^{2+}-permeable AMPA receptors in spinal motoneurons, delayed cytochrome c-release from mitochondria and reduced gliosis.	Tateno *et al.*, 2004
	Tg mice	SOD1 G93A	Increased susceptibility to kainate-induced excitotoxicity. SOD1 G93A mutation causes changes in AMPA-receptor expression and function.	Spalloni *et al.*, 2004
	HEK293	VAPB P56S	VAPB interacts with the outer mitochondrial membrane protein PTPIP51. Loss of either VAPB or PTPIP51 impaired mitochondrial calcium homeostasis.	De Vos *et al.*, 2012

Table 1

Continued

Mitochondria dysfunctions	Model	Gene mutation	Major findings	References
Oxidative stress	Human ALS CSF samples	NA	Increase in 3-nitrotyrosine and 3-nitrotyrosine/ tyrosine ratio.	Tohgi *et al.*, 1999
	Tg mice Knockout mice	SOD1 G93A SOD1$^{-/-}$	Increased ROS production.	Goldstein *et al.*, 2008
	Tg mice	SOD1 G93A	Enhanced oxidative damage of mitochondrial proteins.	Mattiazzi *et al.*, 2002
	Tg mice	SOD1 G93A	Increased oxyradical production, sustained elevations of intracellular calcium levels, and mitochondrial dysfunction.	Kruman *et al.*, 1999
	NSC-34	SOD1 G93A	Depletion of mitochondrial glutathione	Muyderman *et al.*, 2009
	NSC-34	Wt-hTDP-43 Q331K M337V	Increased ROS production.	Hong *et al.*, 2012
	Yeast	TDP-43 Q337K	Increased ROS production	Braun *et al.*, 2011
Apoptosis	NSC-34	SOD1 G85R, A4V	Activated caspase 3, Bax and cytochrome c in cells bearing mutant SOD1 inclusions but not in cells expressing dispersed SOD1.	Soo *et al.*, 2009
	HEK293, SH-SY5Y	SOD1 G93A	Mutant SOD1 induces mitochondrial morphological changes and compromises mitochondrial membrane integrity leading to release of cytochrome C only in the presence of Bcl-2.	Pedrini *et al.*, 2010
	NSC-34 Tg mice	SOD1 G93A	Induced Bcl2-A1 expression via the AP1 transcription factor in motor neuronal cells. Bcl2-A1 interacted with pro-caspase-3, via its C-terminal helix α9, preventing the completion of caspase-3 processing.	Iaccarino *et al.*, 2011
	Tg rats	SOD1 G93A	Decrease in mitochondrial protein import.	Li *et al.*, 2010
	N2A	SOD1 G93A	Both Wt and mutant SOD1 bind the anti-apoptotic protein Bcl-2.	Pasinelli *et al.*, 2004
	Tg mice, Tg rats	SOD1 G93A, H46R, G85R, G127X	Misfolded SOD1 deposited onto the cytoplasmic face of the outer mitochondrial membrane.	Vande *et al.*, 2008
	Tg mice G93A-SOD1/BAX-BAK deleted mice	SOD1 G93A	Neuronal deletion of Bax and Bak halted neuronal loss, prevented axonal degeneration, delayed symptom onset, weight loss and extended survival.	Reyes *et al.*, 2010
	Tg mice	SOD1 G93A	Bid cleavage at the time for disease onset.	Guegan *et al.*, 2002
	Tg mice	SOD1 G93A	A broad caspase inhibitor zVAD-fmk delayed disease onset and mortality.	Li *et al.*, 2000
	Tg mice	Wt-TDP-43	Caspase-3 activation.	Tsai *et al.*, 2010
	Human ALS spinal cord samples		Increased expression of Bax but no change in Bcl-2 and ICH-1 L expressions. Positive for TUNEL staining of motor neurons.	Ekegren *et al.*, 1999

broad caspase inhibitor delays onset and slows disease progression in this transgenic mouse (Li *et al.*, 2000).

Mutant SOD1, mitochondria and oxidative stress

Several recent studies link the presence of mutant SOD to an increased production of free radicals such as peroxynitrite, hydrogen peroxide and hydroxyl radicals (Table 1) (Crow *et al.*, 1997; Beckman *et al.*, 2001; Poon *et al.*, 2005; Rizzardini *et al.*, 2005). It has been suggested that the presence of mutations in SOD1 causes the dissociation of zinc from the enzyme thereby promoting an increase in mitochondrial superoxide production which results in downstream radical formation and oxidative damage (Estevez *et al.*, 1999). Both wild-type and mutant SOD1 also directly interact with the superoxide producing enzyme NADPH oxidase via the RAc1

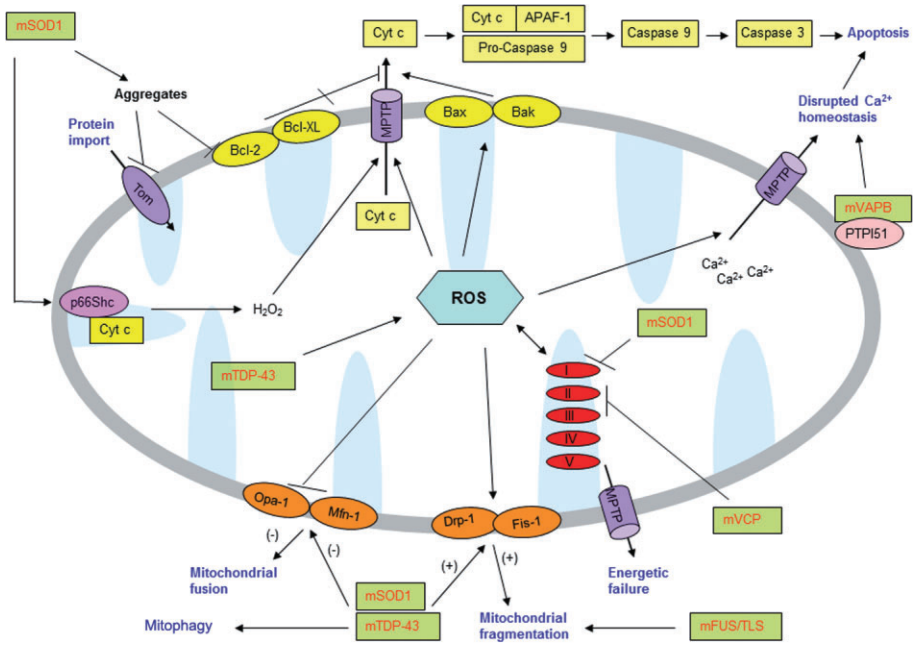

Figure 2

ALS-associated mutant proteins in mitochondrial dysfunction: mutant SOD1 (mSOD) aggregates at the outer membrane of mitochondria, inactivating the anti-apoptosis protein Bcl-2 (Pedrini *et al.*, 2010) resulting in the release of cytochrome c possibly triggering mitochondrial intrinsic apoptosis (Pasinelli *et al.*, 2000; 2004). mutant SOD1 also activates p66Shc at the intermembrane space, inducing cytochrome c oxidation and production of hydrogen peroxide (Pesaresi *et al.*, 2011). mutant SOD1 association with mitochondria also results in other mitochondrial dysfunctions such as: aberrant morphology (Song *et al.*, 2013), impair fusion/fission processes (Liu *et al.*, 2013), impaired protein import (Li *et al.*, 2010), impaired ETC complex activity affecting oxidative phosphorylation (Ferri *et al.*, 2006), impaired calcium homeostasis (Parone *et al.*, 2013) and increased oxidative stress (Mattiazzi *et al.*, 2002; Goldsteins *et al.*, 2008). Abnormal mitochondrial dynamics and fragmentation are also found in mutant TDP-43 (mTDP-43), FUS/TLS (mFUS/TLS) and VCP (mVCP) models (Sasaki *et al.*, 2007; Huang *et al.*, 2011; Xu *et al.*, 2011; Nalbandian *et al.*, 2013; Wang *et al.*, 2013). Both mutant SOD1 and mutant TDP-43 are localized to mitochondria and may induce various degrees of mitochondrial dysfunction (Mattiazzi *et al.*, 2002; Wang *et al.*, 2013). Mutant VAPB (mVAPB) interacting with outer mitochondrial membrane protein PTPIP51 disrupts calcium buffering (De Vos *et al.*, 2012).

protein (Harraz *et al.*, 2008; Polymenidou and Cleveland, 2008). However, mutant SOD1 has a higher affinity for RAc1 than wild-type SOD1 possibly resulting in chronic activation of NADPH oxidase. In support of this finding, a more than 10-fold increase in superoxide production has been reported in cells carrying mutant $SOD1^{G93A}$ compared with cells over-expressing the wild-type form of the protein (Harraz *et al.*, 2008).

Cellular defence against oxidative stress involves SOD itself, catalase and glutathione-dependent processes. Glutathione is mainly localized to the cytoplasm but some is transported into the mitochondria and typically accounts for less than 15% of the total (Griffith and Meister, 1985; Meister, 1995; Fernandez-Checa *et al.*, 1998). Glutathione acts by both directly detoxifying ROS and, acting as a substrate for many different peroxidases (Dringen, 2000; Sims *et al.*, 2004; Sims and Muyderman, 2010). However, glutathione is also involved in other important functions in cells including the modification of exogenous molecules via the actions of glutathione S-transferases (Sheehan *et al.*, 2001) and the reversible glutathionylation of proteins (Giustarini *et al.*, 2004; Shelton *et al.*, 2005). It is important to note that mitochondria lack catalase the emzyme that normally detoxifies hydrogen peroxide. Mitochondrial glutathione plays a major role in reactions catalyzed by glutathione peroxidase that are involved in the removal of mitochondrial peroxides. We have previously demonstrated the importance of mitochondrial glutathione in maintaining viability in several neural cell types and depletion of this antioxidant pool is directly associated with dysfunction and loss of viability in a range of cells challenged with oxidative stress (Sims *et al.*, 2004; Muyderman *et al.*, 2007).

Mitochondrial glutathione in ALS

Although the role of mitochondrial antioxidant defences involving glutathione has been rigorously investigated in other neurological conditions and in other cell types (Sims *et al.*, 2004) the evidence for a role in ALS is mostly indirect. Experimentally decreased glutathione levels speed up disease progression in the mutant $SOD1^{G93A}$ transgenic (Vargas *et al.*, 2011) and loss of total glutathione content in motor neurons has been reported in cell lines carrying the same mutation (Rizzardini *et al.*, 2003). Moreover, reduced levels of oxidized glutathione have been reported in cerebrospinal fluid

from ALS patients (Tohgi *et al.*, 1999). In addition, we have recently shown that the total glutathione content in the mutant SOD1^{G93A} expressing NSC-34 cell is no different from the parent NSC-34 cell. However, these cells had significantly lower levels of mitochondrial glutathione when this pool was measured separately, and this phenomenon was associated with an increased susceptibility to oxidative stress with subsequent apoptotic cell death (Muyderman *et al.*, 2009). Therefore it is plausible to suggest that at the very least, the SOD1^{G93A} mutation induces a low grade of mitochondrial oxidative stress resulting in the consumption of glutathione or the export of oxidised glutathione from the mitochondria thereby making them more susceptible to mitochondrial oxidative stress than their normal counterparts. This hypothesis is consistent with studies in which treatments protecting mitochondria from oxidative stress have shown a highly beneficial effect on the survival of both CNS astrocytes and motor neurons (Cassina *et al.*, 2008). Moreover, overexpression of the mitochondrial antioxidant genes, MnSOD and GPX4, protect against mutant SOD1 in the NSC-34 cell line (Liu *et al.*, 2002). These findings represent some of the most direct evidence to demonstrate the key role of the mitochondrial glutathione pool in preserving viability under pathological conditions. Interestingly, studies involving intraventricular glutathione delivery have been shown to restore mitochondrial glutathione levels after ischaemia and protect against ischaemic damage (Anderson *et al.*, 2004). However, to our knowledge, no attempts have been made to explore this potentially effective therapeutic approach in ALS.

Electron transport chain deficiency in ALS

Mitochondria are responsible for oxidative metabolism. ATP production is dependent on the ETC where complex I (NADH dehydrogenase) is believed to be the rate limiting step (Pathak and Davey, 2008). Although evidence is sparse and many times contradictory, deficiencies in the activities of complex I and II-III (Browne *et al.*, 1998) and complex IV (Menzies *et al.*, 2002; Kirkinezos *et al.*, 2005) have been reported from human tissue as well as from animal and cell culture models. In contrast, however a marked increase in complex I activity has been demonstrated in *post mortem* material (Bowling *et al.*, 1993). Moreover, platelet mitochondria from ALS patients are able to restore fully ETC activity in Rho-zero cells who lack these organelles (Gajewski *et al.*, 2003). On the other hand, spinal cord mitochondria isolated from transgenic mutant SOD1 mice show decrease in the activity of several complexes which is associated with decreased oxygen consumption and ATP production (Mattiazzi *et al.*, 2002). Consistent with this observation, expression of mutant SOD1 in neuroblastoma-derived SH-SY5Y leads to loss of mitochondrial membrane potential (Carri *et al.*, 1997). Moreover decreases in complex IV activity associated with a loss of cytochrome c have been reported in mitochondria isolated from presymptomatic transgenic mutant SOD1 mice (Kirkinezos *et al.*, 2005).

Mitochondrial permeability transition pore in ALS

Involvement of the mitochondrial permeability transition pore has been implicated in mitochondrial dysfunction in several studies of ALS. Proteins responsible for pore formation are highly expressed in motor neurons and cyclophilin D (a matrix protein with peptidyl-prolyl cis-trans isomerase activity with a key role in the development of the transition pore) is found in high concentrations in swollen mitochondria in the SOD1 animal model. The role of permeability transition pore formation is further supported from studies in which silencing of cyclophilin D expression resulted in delayed disease onset and increased survival in the mutant SOD1 mouse. Interestingly, in an attempt to investigate the role of calcium overload, Parone *et al.* (2013) found that eliminating cyclophilin D in the mutant SOD1 mouse resulted in a significantly increased calcium mitochondrial buffering capacity and improved oxidative metabolism associated with normalised mitochondrial morphology and reduced mitochondrial swelling (Table 1). However, despite significantly reduced motor neuron death, both disease progression and lifespan were unaltered suggesting that other non-mitochondrial processes are the primary contributors to motor neuron cell death in this model (Parone *et al.*, 2013). In contrast, earlier studies demonstrated a beneficial effect of interfering with the permeability transition in similar mutant SOD1 mouse models. For example, cyclosporin A, which inhibits transition pore opening by binding to cyclophilin D, slowed disease progression and increased lifespan when administered to SOD1 transgenic mice (Karlsson *et al.*, 2004). In another study, it was shown that FK506 that mimics some effects of cyclosporin but lack effects on the permeability transition, did not affect survival in the same transgenic mouse model (Anneser *et al.*, 2001). Together these studies strongly indicate a role for the permeability transition pore in ALS; however, it should be noted that the effects on lifespan were modest and that motor neuron death eventually occurred in spite of the absence of transition pore formation. Hence, other mechanisms leading to mitochondrial dysfunction and the development of cell death must occur in parallel.

It has been suggested that oxidative stress or calcium overload are triggers for transition pore opening in ALS (Martin *et al.*, 2009). As pore formation is sometimes associated with necrosis-like cell death, it is possible that the permeability transition is secondary to, or results from, a decline in energy metabolism, or alternatively that transient induction of the pore initiates changes leading to a more slowly developing programmed form of necrosis. A likely explanation for this observation could be that the final development of caspase-dependent apoptosis is overwhelmed by other molecular changes that result in these alternative forms of cell death, a view that would be consistent with findings of low ATP/ADP ratio and impairment of the ETC activity reported in cells from ALS patients.

New ALS mutations – new views

The rapid advances in the understanding of ALS pathology over the last decade have considerably changed our view of

the pathogenesis of this disease. It is now believed that impaired RNA metabolism produce similar, if not identical, disease phenotypes as mutations in the SOD1 gene. This raises the question whether as mitochondrial dysfunction is exclusive to mutant SOD1 toxicity or if it can be applied to newly discovered forms of fALS. Even though the evidence is still indirect, several findings point in the direction of impaired mitochondrial function, at least in TDP-43 pathology, playing a central role to the overall pathogenesis of ALS (see Figure 2: Table 1).

TDP-43 is a highly conserved 43-kDa DNA- and RNA-binding protein regulating transcription and splicing (Bose *et al.*, 2008). The protein is abundantly expressed in neurons and glia. TDP-43 predominantly localizes in nuclei, but is also found in cytosolic stress granules where it is believed to participate in local post-transcriptional modifications of mRNA and translational control (Buratti and Baralle, 2008). TDP-43 has a promiscuous protein interaction pattern with more than 200 targets reported, suggesting an involvement in a vast array of intracellular events (Freibaum *et al.*, 2010). The physiological role of TDP-43 is, to a large extent, unknown although it has been suggested to act as a neuronal response factor and to play a prominent role in the stability of neurofilament mRNA and in microRNA biogenesis (Wang *et al.*, 2008; Volkening *et al.*, 2009). Abnormal processing and aggregation of TDP-43 is a characteristic of so called TDP-43 proteinopathies (Kwong *et al.*, 2007). In these disorders, TDP-43 is relocated from the nuclei to the cytoplasm and sequestered into inclusions mainly composed of ubiquitinated and phosphorylated C-terminally truncated fragments (Neumann *et al.*, 2006; Geser *et al.*, 2008). Abnormal molecular weight TDP-43 fragments have been observed in neurons and astrocytes in patients from a spectrum of neurodegenerative diseases including 95% of familial and sporadic ALS (Mackenzie *et al.*, 2007; Liscic *et al.*, 2008) making it an interesting candidate for all forms of the disease.

It is currently not known if mutations in TDP-43 affect post-transcriptional modifications of gene products that control or modify mitochondrial function or if mitochondrial dysfunction in TDP-43 proteinopathies result from direct toxic effects by truncated and aggregated TDP-43. Nevertheless, transgenic animals display mitochondrial changes that resemble some of those found in human ALS. In non-vertebrate models, mutated TDP-43 aggregates around mitochondria and has been shown to cause negative effects on oxidative metabolism (Braun *et al.*, 2011). Moreover, transgenic animals expressing mutant TDP-43 exhibit changes in mitochondrial trafficking, mitochondrial clustering, vacuolization and the expression of proteins responsible for mitochondrial fusion and fission (Figure 2: Table 1) (Shan *et al.*, 2010; Xu *et al.*, 2010; Wang *et al.*, 2013). Interestingly, it seems that the presence of mutant TDP-43 may not be the only mechanism by which mitochondrial damage is induced in TDP-43 pathology. In a recent study, Hong *et al.* (2012) demonstrated that overexpression of wild-type TDP-43 results in mitochondrial damage and that this is associated with activation of mitophagy. In this study, both truncated and full-length TDP-43 was localized to the mitochondria thereby supporting the view of a direct toxic effect on these organelles, possibly mediated by impaired autoregulation of TDP-43 protein expression. These findings are important, as impaired TDP-43 homeostasis is likely to occur in sporadic occurring cases of ALS in which no genetic cause of the pathology can be found.

Mutations in the RNA binding protein FUS have also recently been linked to the pathogenesis of ALS although FUS is involved in different aspects of RNA metabolism than that of TDP-43. In FUS proteinopathies, the protein is believed to translocate to the cytoplasm causing ER stress with possible downstream effects on mitochondrial function (Farg *et al.*, 2013). Little is known about how mutations in FUS affect mitochondrial function, however, transgenic rats overexpressing mutant FUS display motor neuron death with features of mitochondrial dysfunction (Huang *et al.*, 2011). The introduction of a FUS mutation in murine spinal cord motor neuron cultures resulted in cell death and changes in mitochondrial morphology similar to those reported in the SOD1 animal model (Tradewell *et al.*, 2012). Moreover, indirect evidence of mitochondrial dysfunction has been reported in *post mortem* tissue from one patient suffering from juvenile ALS with mutations in FUS as the only pathogenic factor (Huang *et al.*, 2010). The recent advances in identifying new genes responsible for the pathogenesis in ALS suggest that genes encoding for proteins linked to rare cases of ALS such as UBQLN2, SQTM1/p62, VAPB and VCP, may all result in a common pathogenic pathway involving mitochondrial dysfunction (see Figure 2). Interactions between these proteins and those of the more common proteinopathies in ALS need to be investigated further.

Together these data are all indicative of mitochondrial dysfunction in non-SOD1 ALS but further studies are required to provide enough data to make pharmacological interventions possible. Thus it seems plausible to conclude that altered RNA metabolism has the potential to disrupt normal mitochondrial function.

Targeting mitochondria in the treatment of ALS

Rilazole is currently the only approved drug for the treatment of ALS but the compound only exerts modest effects on survival and is without effect on motor function (Bensimon *et al.*, 1994; Hugon, 1996). Overall, the mean increase in survival is approximately 3 months (Miller *et al.*, 2012). Rilazole has many potential targets but is believed to exert its main function by reducing presynaptic glutamate release and thereby reducing excitotoxicity. There have been numerous clinical trials over the last decades targeting mechanisms predominantly identified in SOD1 transgenic animals including protein clearing agents such as lithium (Miller *et al.*, 2011); analeptic drugs such as topiramate which reduces extracellular glutamate (Cudkowicz *et al.*, 2003); anti-inflammatory drugs primarily targeting glial activation such as minocycline (Gordon *et al.*, 2007) and the broader 5-HT_{1A} receptor agonist, xaliproden, a drug with proven neurotrophic and neuroprotective activities (Meininger *et al.*, 2004). Unfortunately, these single drug approaches have proven ineffective.

There are many problems associated with identifying a single pharmacological target in ALS. Firstly, the ethology of

the disease is still, to a large extent, unknown and the disease is relatively rare making large clinical trials difficult. The complex pathogenesis of ALS raises two possible explanations on why previous pharmacological approaches have failed when translated to humans: (i) findings from the SOD1 models may not apply to all cases of ALS, especially considering that 90% of patients present with TDP-43 pathology which is absent in patients with SOD1 mutations. This observation highlights the need for studies involving newly discovered ALS-causing mutations such as those of TDP-43, FUS and the C9orf72 hexanucleotide repeat expansion. (ii) The multiple pathogenic processes demonstrated in ALS support the view that multiple pathways are converging to a common endpoint resulting in motor neuron loss. This may also explain the disappointing results of targeting a single pathological process such as caspase inhibitors. As neuronal loss in ALS is most likely to reflect combinations of multiple pathogenic mechanisms including glutamate excitoxicity, oxidative stress, mitochondrial dysfunction and disruption of axonal transport processes, a multidrug approach is most likely to be needed. In addition, activation of glial cells results in secretion of pro-inflammatory cytokines resulting in further toxicity and glial cells expressing ALS-linked mutations are known to have detrimental effects on surrounding motor neurons highlighting the need for glia-directed pharmacological interventions.

In developing a multidrug approach, individual targets must be identified. Considering the vast amount of evidence of mitochondrial dysfunction in both the SOD1 model and in models of the later discovered genetic mutations, maintaining mitochondrial function is an appealing part of such an approach. There have been several studies addressing the role of pharmacological interventions targeted at mitochondrial function although the outcomes have been limited. One such drug is pramipexole (PPX). PPX is a dopamine analogue believed to exert neuroprotective effects through enhancing mitochondrial function and inhibiting activation of mitochondrial linked apoptotic pathways at the same time counteracting glutamate-mediated excitotoxicity (Alavian *et al.*, 2012). PPX also scavenges reactive oxygen species and lowers free radical levels in ALS patients (Pattee *et al.*, 2003). Dexpramipexole is the R(+) enantiomer of PPX and is tolerated in higher doses than PPX and both have shown promise in early clinical studies (Cudkowicz *et al.*, 2011). Dexpramipexole has similar neuroprotective properties to that of PPX but lacks dopaminergic effects. Most likely, both PPX and Dexpramipexole, exert part of their neuroprotective effects though direct effects on mitochondria by stabilizing the proton gradients needed for ATP production. Dexpramipexole was recently shown to decrease mortality in a Phase 2 study in subjects with ALS (Rudnicki *et al.*, 2013) making this an interesting candidate in a multidrug approach for the treatment of ALS.

Melatonin is another compound that recently gained interest by its mitochondria stabilizing effects in ALS. In the mutant SOD1^{G93A} model, melatonin decreases cytochrome c release, caspase-3 activation and delays disease progression (Zhang *et al.*, 2013). Moreover, treatment with melatonin also resulted in a reduction in the activation of astrocytes and microglia indicating an attenuated inflammatory response. Moreover, treatment with SOD1-like peptides has been shown to restore mitochondrial function in mutant SOD1^{G93A} mice (Tan *et al.*, 2013).

A possible drug target associated with mitochondrial function is the transcriptional co-activator PPARγ co-activator-1α (PGC-1α). PCG-1α is a regulator of mitochondrial oxidative metabolism and is activated by several converging pathways including cAMP and cytokines (Puigserver and Spiegelman, 2003) and reduced PCG-1α mRNA levels have been described in ALS patients (Thau *et al.*, 2012). Overexpressing PCG-1α in the SOD1^{G93A} model improves motor function and prolongs survival possibly by restoring close to normal ETC activity (Zhao *et al.*, 2011). Interestingly, PCG-1α/G93A animals also show an increased expression of astrocytic GLT-1 glutamate transporters that may have been the ultimate cause of neuroprotection seen in this model. Whether this is a primary or secondary event is not known and the relation to enhanced mitochondrial function is unclear. In the context of ETC activity, treatment with creatine has been shown to enhance mitochondrial activity and slow disease progression in the G93A SOD1 model (Klivenyi *et al.*, 2004); however, clinical trials have so far been disappointing (Shefner *et al.*, 2004). Although many of these studies have shown potential to increase lifespan and delay disease onset, the effects are in general modest and highlights the need for larger multidrug studies in non-SOD1 models of the disease.

Concluding remarks

Evidence of mitochondrial dysfunction in ALS has been reported for more than 50 years and is today recognized as central in the disease process. However, it is still unclear if changes in mitochondrial biology is a primary event in the pathology or if it occurs secondary to other cellular processes resulting in oxidative stress and activation of apoptotic pathways. Nevertheless, the extent of preservation of key mitochondrial properties and the ability of these organelles to mount appropriate defensive responses must still be regarded as important determinants of tissue viability in ALS and hence is a strong indicator for mitochondrial-directed pharmacological interventions. Based on the current evidence of multifaceted mitochondrial dysfunction reported in human ALS and in most rodent animal models, it seems obvious that pharmacological interventions targeting mitochondrial function are essential for future treatment strategies in ALS Interventions that restore, or promote mitochondrial function seem a plausible approach, however, based on the data reviewed above it is unlikely that ALS is solely a mitochondrial disease and that any pharmacological intervention may have to have multiple targets, possibly including those in non-neuronal cells types as astrocytes and microglia. The time of a single drug approach for treatment of ALS is gone but mitochondria are still a valid target.

Conflict of interests

None.

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