Mitochondrial dysfunction in a cell culture model of familial amyotrophic lateral sclerosis

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Summary

The molecular mechanisms by which mutations in the gene for Cu/Zn superoxide dismutase (SOD1) lead to the selective death of motor neurones in familial amyotrophic lateral sclerosis (FALS) remain incompletely understood. Previous evidence has indicated that mitochondrial abnormalities may develop during motor neurone injury, but several important questions remain unanswered. We have developed a cell culture model of FALS in which a motor neurone cell line (NSC34) has been stably transfected to express normal or mutant human SOD1 at levels approximating to those seen in the human disease. The aims of the study were to: (i) investigate whether morphological mitochondrial abnormalities occur at expression levels of mutant SOD1 close to physiological levels; and (ii) determine whether the presence of mutant SOD1 causes abnormalities of mitochondrial respiratory chain function and changes in cellular bioenergetic parameters in motor neuronal cells. Using this cellular model, we demonstrate that the presence of mutant SOD1 results in the development of abnormally swollen and pale staining mitochondria. These morphological changes are accompanied by biochemical abnormalities with specific decreases in the activities of complexes II and IV of the mitochondrial electron transfer chain. These same complexes are inhibited when control NSC34 cells are subjected to oxidative stress induced by serum withdrawal. The decrease in respiratory chain complex activity in the presence of mutant SOD1 was not accompanied by decreased expression of representative proteins present in these complexes. Motor neuronal cells expressing mutant SOD1 showed increased cell death when exposed to oxidative stress by serum withdrawal, whereas the presence of normal human SOD1 exerted a protective effect. Under basal, unstressed culture conditions, no change in the ATP : ADP ratio was observed in the presence of mutant SOD1. However, the mitochondrial changes associated with the presence of mutant SOD1 clearly had adverse cellular bioenergetic consequences as shown by increased cell death in the presence of pharmacological inhibition of the glycolytic pathway. We conclude that one important mechanism by which mutant SOD1 causes motor neurone injury involves inhibition of specific components of the mitochondrial electron transfer chain. Therapeutic measures aimed at protecting mitochondrial respiratory chain function may be useful in SOD1 related familial and possibly other forms of amyotrophic lateral sclerosis.

Keywords: Cu/Zn superoxide dismutase; mitochondria; oxidative stress; motor neurones; amyotrophic lateral sclerosis

Abbreviations: ALS = amyotrophic lateral sclerosis; FALS = familial amyotrophic lateral sclerosis; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SOD1 = Cu/Zn superoxide dismutase

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease that produces progressive muscle weakness and wasting of the limb and bulbar muscles and eventual death from respiratory failure in most cases. The cell

death process in ALS is relatively selective for lower motor neurone groups in the spinal cord and brainstem, and for upper motor neurones in the motor cortex. The precise molecular pathways leading to motor neurone injury and cell death in ALS remain incompletely understood. A body of evidence has emerged to indicate that the neuronal injury in ALS reflects a complex interaction between genetic factors, imbalance of the glutamatergic transmitter system and oxidative stress (Brown, 1995; Shaw and Ince, 1997; Cookson and Shaw, 1999; Andersen *et al.*, 2000; Cleveland and Liu, 2000). There is also emerging evidence that motor neurones may die by a programmed cell death pathway (Sathasivam *et al.*, 2001). In relation to several of these factors, there are cell-specific features which may render motor neurones susceptible to injury (Shaw and Eggett, 2000).

In ~20% of familial cases, or 2% of the ALS population as a whole, mutations in the gene encoding the free radical scavenging enzyme Cu/Zn superoxide dismutase (SOD1) have been identified (Rosen *et al.*, 1993). There is robust evidence that mutant SOD1 exerts its deleterious effects through a toxic gain of function rather than through a loss of superoxide dismutase activity. The major hypotheses for the toxicity of mutant SOD1, which are not mutually exclusive, include: alteration in the handling of intracellular free radicals leading to increased formation of damaging hydroxyl radicals (Wiedau-Pazos *et al.*, 1996; Yim *et al.*, 1996; Liu *et al.*, 1999; Said Ahmed *et al.*, 2000) and peroxynitrite derivatives (Beckman *et al.*, 1993); and misfolding of the mutant protein leading to the formation of intracellular SOD1 aggregates (Shibata *et al.*, 1996; Bruijn *et al.*, 1998; Johnston *et al.*, 2000).

In both the subtype of ALS associated with SOD1 mutations and in the sporadic disease, there has been considerable recent interest in the possibility that mitochondrial damage may contribute to age-related motor neuronal injury (Beal, 1998; Cortopassi and Wong, 1999; Menzies *et al.*, 2002). Important properties of mitochondria relevant to the pathogenesis of neurodegenerative diseases include the generation of intracellular ATP, the buffering of intracellular calcium, the generation of intracellular free radical species and involvement in the initiation of programmed cell death or apoptosis. Mitochondrial proteins and DNA have been shown to be particularly susceptible to oxidative stress and free radicals are known to inhibit the activities of specific mitochondrial enzymes (Zhang *et al.*, 1990; Radi *et al.*, 1997). Studies in human post-mortem CNS tissue have been reported to show ultrastructural changes in mitochondrial morphology in ALS (Sasaki and Iwata, 1996). Alterations in the activities of respiratory chain complexes have been described in sporadic ALS, showing increased activity of complexes I and II/III in the frontal cortex of patients with SOD1-related ALS (Bowling *et al.*, 1993), and decreased complex IV activity in the spinal cord (Fujita *et al.*, 1996) and individual spinal motor neurones (Borthwick *et al.*, 1999). In addition to these enzyme activity studies, levels of the

'common deletion' (a 4977 base pair deletion of mitochondrial DNA) are increased in the motor cortex of ALS cases compared with controls (Dhaliwal and Grewal, 2000).

Further evidence that mitochondrial dysfunction may play a contributory role in motor neurone injury has arisen from the study of cellular and animal experimental models. A neuronal cell line transfected to express mutant SOD1 was reported to show loss of mitochondrial membrane potential, together with an increase in cytosolic calcium, suggesting a reduction in the ability of mitochondria in these cells to sequester calcium (Carri *et al.*, 1997). In transgenic mouse models of SOD1 related ALS, one of the very early features of motor neurone injury occurring before the animals develop clinical signs of motor dysfunction is the appearance of morphologically abnormal mitochondria, which become swollen and eventually form vacuolar structures (Kong and Xu, 1998).

These data from human CNS tissue and experimental models clearly suggest that mitochondria may represent a subcellular organelle susceptible to damage in ALS. However, the data currently available in this research field leave several important questions unanswered, which could potentially be addressed using a cellular model of SOD1-related motor neurone injury. These include:

(i) Does the presence of mutant SOD1 cause morphological abnormalities of mitochondria when expressed at physiological levels or only when there is a marked over-expression of the mutant protein as seen in the SOD1 transgenic mouse models?

(ii) Are the morphological changes observed in mitochondria in transgenic mice harbouring mutant SOD1 functionally significant and accompanied by detectable biochemical changes?

(iii) What are the functional consequences of any changes in mitochondrial function in the presence of mutant SOD1?

The present study aimed to address these three questions using a motor neurone cell line (NSC34) transfected to express normal or mutant human SOD1 at levels approximating to those seen in the human disease. The NSC34 cell line is a mouse motor neurone/neuroblastoma cell line which has the advantage of retaining certain motor neurone like properties (Cashman *et al.*, 1992), and which can be propagated at sufficient density in culture to allow biochemical changes to be measured.

Material and methods
Materials
General reagents, donkey anti-sheep secondary antibody and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma (Poole, Dorset, UK). Medium and serum for tissue culture was purchased from Gibco (Paisley, UK). DOSPER and hygromycin B were from Boehringer Mannheim (Lewes, UK). Electron microscopy reagents were purchased from TAAB (Aldermaston, Berkshire,

UK). Sheep polyclonal antibody to SOD1 was from the Binding Site (Birmingham, UK). Reagents for enhanced chemiluminescence (ECL) detection of Western blots were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Tissue culture plastics were from Greiner (Stonehouse, Gloucestershire, UK). The Coomassie Blue protein assay kit was from Pierce (Chester, Cheshire, UK).

Generation, maintenance and characterization of SOD1 transfected cell lines

The generation of pCEP4 expression vectors containing either normal or mutant forms of human SOD1 has been described elsewhere (Durham et al., 1997). In this study, the effects of two SOD1 point mutations were investigated, i.e. glycine 93 → alanine (G93A) and glycine 37 → arginine (G37R). Control cell lines consisted of cells transfected with normal human SOD1 or the vector alone. NSC34 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) as described previously (Cookson et al., 1998). For transfections, cells were plated overnight at a density of 1×10^4 cells/cm^2 in 12 well multiplates. DNA (0.5 µg/well) was transfected into the cells using the cationic liposomal reagent DOSPER (1.5 µg/well). After 24 h, cells were selected for resistance to 300 µg/ml hygromycin for 2 weeks and subsequently maintained in the presence of 300 µg/ml hygromycin. Limiting dilution was used to generate individual clonal cell lines.

Hygromycin-resistant cell lines were then screened for expression of SOD1 protein by Western blotting. Total cell proteins were extracted as previously described (Cookson et al., 1998). Ten micrograms of total protein per sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide gels) and transferred to Immobilon-P membranes (Millipore, Watford, UK). Blots were blocked in TBS-T [20 mM Tris–HCl pH 7.6 plus 137 mM NaCl and 0.1% (v/v) Tween-20] and 5% (w/v) dried skimmed milk. They were then probed with polyclonal anti-SOD1 diluted 1 : 2000 in TBS-T plus milk (overnight at 4°C), followed by peroxidase-conjugated secondary antibody (1 : 1000, 1 h at room temperature). Antibody binding was revealed using enhanced chemiluminescence according to the manufacturer's instructions.

The continued expression of human SOD1 was checked on a weekly basis to ensure that the expression vectors were retained within the cells. To limit any potential effects of senescence, cells were never maintained beyond passage 25. No decrease in cell viability in any of the cell lines was apparent during this time under normal basal culture conditions. Cell lines were cultured simultaneously and for each experiment; the different cell lines were harvested at the same passage number to ensure fair comparison. In addition, repeat experiments were carried out at a range of passage numbers.

Electron microscopy

For electron microscopy, NSC34 cells transfected with vector only, normal SOD1 and G93A mutant SOD1 were grown in a T75 flask until approaching confluency. Cells were washed and harvested into phosphate buffered saline (PBS) and centrifuged (600 g, 5 min). The pellet was resuspended in fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate) and fixed for 30 min at room temperature. Following washing with 0.1 M sodium cacodylate, cells were pelleted and resuspended in molten 3% agarose, which was then allowed to set for ease of further processing. Cells in agarose were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate, then washed in 50% ethanol before en bloc staining with 2% uranyl acetate in 50% ethanol. Cells were then dehydrated through a graded series of ethanol and propylene oxide before embedding in resin (epon 812 resin) and polymerized overnight at 60°C. Sections of 70 nm were taken and stained with lead citrate (Reynolds, 1963) and viewed using a transmission electron microscope.

Mitochondrial respiratory chain enzyme activities

Spectrophotometric assays were used to measure the activity of each of the components of the electron transfer chain: complex I (NADH:ubiquinone oxidoreductase); complex II (succinate:ubiquinone oxidoreductase); complex III (ubiquinol:cytochrome c oxidoreductase); and complex IV (cytochrome c oxidase). Assays were carried out on mitochondria isolated from cells after culturing under basal conditions in the presence of 10% serum and at time points 6 h, 24 h and 48 h following oxidative stress induced by serum withdrawal. Cells were harvested and washed in PBS, then pelleted and resuspended in 250 mM sucrose with 2 mM HEPES and 0.1 mM ethylene glycol tetraacetic acid (EGTA) pH 7.4, before being homogenized using a glass–Teflon homogenizer. The homogenate was centrifuged at 3000 r.p.m. (Sorvall SS-34 rotor, Kendoo Laboratory Products Ltd, Bishop's Stortford, UK) for 6 min and the pellet was rehomogenized and recentrifuged. The supernatant from both these steps was combined and the mitochondria pelleted by centrifugation at 12 500 r.p.m. for 10 min. This pellet was washed and finally resuspended in buffer, as above, before snap freezing in 20 µl aliquots and storing at –80°C until use. Protein concentration was determined in mitochondrial preparations using a Coomassie Blue protein assay.

Enzyme activity assays were carried out as described previously (Taylor and Turnbull, 1997). Briefly, complex I was measured as a decrease in absorbance at 340 nm due to the oxidation of NADH with a reference wavelength of 425 nm. Complex II measurements followed the succinate-dependent reduction of 2,6-dichlorophenol-indophenol at 600 nm. Complex III was measured by following the reduction of cytochrome c (III) by ubiquinol at 550 nm with a reference wavelength of 580 nm. Complex IV was also

measured at 550 nm (with a reference wavelength of 580 nm) following the oxidation of cytochrome *c* (II). All enzyme activities are shown corrected for the amount of protein in the preparation as described previously (Parker *et al.*, 1994; Swerdlow *et al.*, 1998; Schapira, 1999). Measurements were made from three independent mitochondria preparations ($n = 3$) and, for each preparation, complex activity was calculated as the mean of at least three repeated measurements.

Protein expression levels of respiratory chain complexes showing altered biochemical activity

Western blotting was used to determine whether any of the changes in activity of respiratory chain complexes were accompanied by alterations in the level of overall protein expression. Western blotting was carried out as described above. Complex II was detected using an antibody to the 30 kDa iron/sulphur subunit of succinate dehydrogenase and complex IV using an antibody to subunit 1 of cytochrome *c* oxidase (clone 1 D6-EI-A8, Molecular Probes, Leiden, The Netherlands). Following detection of the complex II and IV subunits, the blots were stripped (62.4 mM Tris–HCl pH 6.7, 2% SDS, 0.5% v/v β-mercaptoethanol, 50°C, 30 min) and then, to control for any variation in the amount of protein loaded onto the gels, the blots were reprobed for actin (primary antibody from Sigma, clone AC-40, 1 : 2000 dilution; secondary antibody, rabbit anti-mouse from Dako, Ely, UK, 1 : 1000 dilution). Blots were photographed and densitometry carried out using AlphaInnotech software (Flowgen, Lichfield, Staffordshire, UK). Values are shown as a ratio of each complex to actin.

Cell viability assays

NSC34 cell lines were seeded into 24 well plates at 4×10^3 cells/well for 7 days in DMEM plus FCS prior to assay. For serum deprivation experiments, the culture medium was replaced by DMEM without FCS, and cell viability monitored after 6 h, 24 h and 48 h. MTT assays for total viable cell number were performed as described previously (Cookson *et al.*, 1998). In each experiment, mean cell viability was calculated from four MTT readings and the data shown represent combined results from three independent experiments for each cell line.

Cellular bioenergetics

ATP/ADP assays

Cells for ATP/ADP assays were seeded at 5×10^5 cells in a T75 flask and cultured for 5 days. After 3 days, medium on cells was changed and replaced with either medium containing serum or serum-free medium for 48 h. The medium was then replaced again 1 h prior to extraction. Extractions were carried out on ice at 4°C. The method used is based on that described by James *et al.* (1999). Cells were washed twice in PBS and then harvested into 1.5 ml PBS. Samples were deproteinized by addition of 1 ml of cell suspension to 1 ml of perchloric acid [6% (v/v) perchloric acid, 4 mM EDTA, 1% Triton X-100] vortexed, incubated for 5 min on ice and then vortexed again before snap freezing.

To measure ATP levels, samples were neutralized in potassium hydroxide solution (2 M KOH, 2 mM EDTA, 50 mM MOPS). After 10 min incubation on ice, samples were centrifuged (10 000 *g*, 30 s) and 50 µl of supernatant added to 400 ml of assay buffer (100 mM Tris, 2 mM EDTA, 50 mM MgCl$_2$, pH 7.75 with glacial acetic acid). Following a further 10 min incubation period at 25°C, 50 µl of substrate [2.4 µg/ml luciferase, 150 µM luciferin, 25% (v/v) glycerol, 0.4 mg/ml bovine serum albumin, 7.5 mM dithiothreitol, in assay buffer] was added and luminescence measured.

In order to measure ADP levels, ATP was first removed from the samples by incubating 100 µl of neutralized sample with ATP sulfurylase [60 µg/ml ATP sulfurylase, 5 mM GMP, 20 mM Na$_2$MoO$_4$ in Tris–HCl buffer (100 mM Tris–HCl, 10 mM MgCl$_2$, pH 8)] for 20 min at 30°C; samples were then centrifuged at 10 000 *g* for 1 min. ATP sulfurylase was inactivated by heating at 100°C for 5 min before samples were cooled on ice and then centrifuged for 1 min at 10 000 *g*. ADP was then converted to ATP by incubation of 100 µl of sample with 10 µl phospho(enol)pyruvate/pyruvate kinase solution (50 µl pyruvate kinase suspension in ammonium sulphate (1667 U/ml, Sigma) with 350 µl 114 mM PEP) in 400 µl Tris-acetate buffer for 10 min at 25°C. ATP was then measured as above. The effectiveness of removal of ATP by ATP sulfurylase was checked by assaying ATP levels without prior incubation with phospho(enol)pyruvate/pyruvate kinase. Known ATP and ADP concentrations were also measured to allow the assay to be calibrated.

Effect of inhibition of glycolysis by iodoacetate

Iodoacetate was used to inhibit glycolysis in cells— following which any ATP generation by the cells must be a consequence of oxidative phosphorylation. For analysis of the effect of iodoacetate, cells were incubated in serum-free medium containing iodoacetate for 5 h at concentrations between 10 µM and 1 mM. In preliminary experiments, a concentration of 30 µM was shown to result in ~50% cell death over this period (data not shown) and this concentration was chosen for use in further experiments. Viability was assessed by MTT assay. Results shown are combined from five independent experiments with four MTT readings for each treatment in each experiment.

Statistics

Changes in cell viability following serum withdrawal and differences in mitochondrial respiratory chain enzyme activities were analysed using one-way ANOVA (analysis of variance) with Newman–Keuls *post hoc* tests to compare individual cell lines. For the effects of serum withdrawal, a

post test for linear trend was employed. The cut-off point for statistical significance was $P < 0.05$.

Results

The NSC34 cell lines were screened for the presence of human SOD1, which migrates at a slower rate than mouse SOD1 in SDS–PAGE gels (Hoffman *et al.*, 1996). In cells transfected with pCEP4 vector containing human SOD1, there was an additional band corresponding to human SOD1, as well the band corresponding to mouse SOD1 (Fig. 1). The levels of expression of the human SOD1 protein were 30–50% of the level of endogenous murine SOD1, as measured by densitometry.

Alteration in the morphology of mitochondria in NSC34 cells in the presence of mutant SOD1

The morphology of the mitochondria in the presence of mutant SOD1 was investigated by electron microscopy. In both NSC34 cells transfected with vector alone and in those expressing normal human SOD1, the mitochondria were seen

Fig. 1 Expression of human SOD1 in NSC34 cells. Western blotting was performed using a polyclonal antibody to SOD1, which recognizes both mouse SOD1 (lower band) and human SOD1 (upper band). Lanes 1–5 represent samples from untransfected cells (wt, lane 1) and stable cell lines transfected with pCEP4 vector (vector, lane 2), normal SOD1 (lane 3) or the G93A (lane 4) and G37R (lane 5) mutant forms of SOD1. The level of human SOD1 protein expressed in these cell lines is approximately 30–50% of the level of the endogenous murine SOD1 protein.

to be densely staining, with cristae which were clearly defined, though sometimes irregular (Fig. 2). In cells expressing G93A mutant SOD1, the mitochondria appeared very different to those in the control cell lines. In >50% of these cells, the mitochondria appeared swollen and vacuolated (Fig. 2). The outer membrane was usually still intact and the remnants of cristae were visible. The differences in mitochondrial morphology were sufficient to allow a blinded observer to identify G93A transfected cell lines from control cell lines. This observation was consistent following two independent preparations that each included two separate clonal cell lines expressing G93A mutant SOD1.

Presence of mutant SOD1 results in a decrease in the activities of complex II and IV of the mitochondrial electron transfer chain in basal cell culture conditions

The results of the measurements of the activities of the mitochondrial electron transfer chain complex activities are shown in Table 1. The presence of mutant SOD1 in NSC34
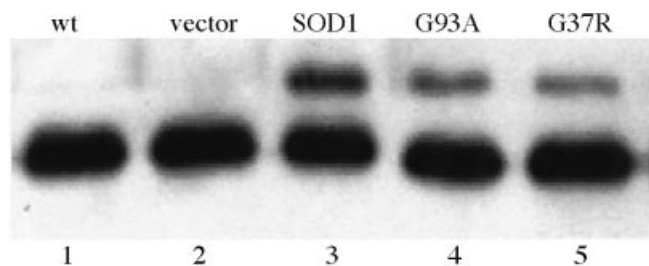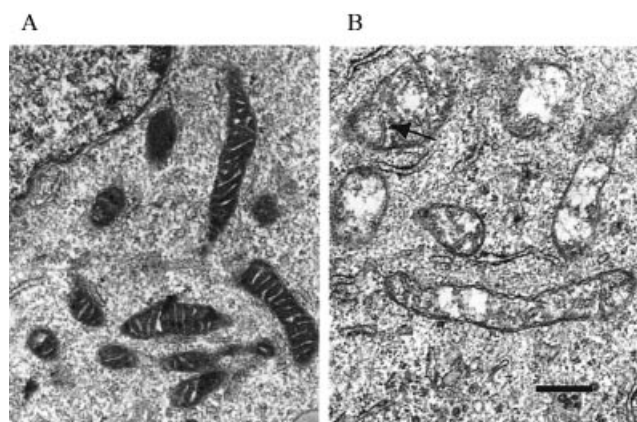

Fig. 2 Altered mitochondrial morphology in the presence of G93A mutant SOD1. (**A**) Mitochondria in vector transfected cells. (**B**) Mitochondria from G93A transfected cells. In the presence of mutant SOD1, the mitochondria are swollen and paler staining, but cristae are still visible within them (indicated by arrow). Scale bar represents 500 nm.

Table 1 *Mitochondrial electron transfer activity in NSC34 cells*

	Complex I (μM/min/mg)	Complex II (μM/min/mg)	Complex III (k/s/mg)	Complex IV (k/s/mg)
Vector	0.16 ± 0.03	0.42 ± 0.05	2.83 ± 0.62	6.38 ± 0.42
SOD1	0.04 ± 0.01	0.33 ± 0.02	4.65 ± 0.93	4.66 ± 0.23
G93A	0.17 ± 0.04	0.23 ± 0.01*#	4.39 ± 0.81	3.60 ± 0.15**##
G37R	0.12 ± 0.02	0.24 ± 0.01*#	3.24 ± 0.89	3.22 ± 0.12**##

Complex activity is shown $\pm$ standard error of the mean in NSC34 cells transfected with empty vector (Vector) or expressing normal human SOD1 (SOD1), G93A mutant human SOD1 (G93A) or G37R mutant human SOD1 (G37R). Measurements were made from three independent mitochondria preparations; for each preparation, complex activity was calculated as the mean of at least three repeated measurements. Statistical analysis was by ANOVA with Newman–Keuls *post hoc* test. *$P < 0.01$; **$P < 0.001$ for mutant cell lines compared with vector transfected cells; #$P < 0.05$; ##$P < 0.01$ for mutant cell lines compared with normal SOD1 transfected cells. There were no statistically significant changes in complex I or complex III activity.
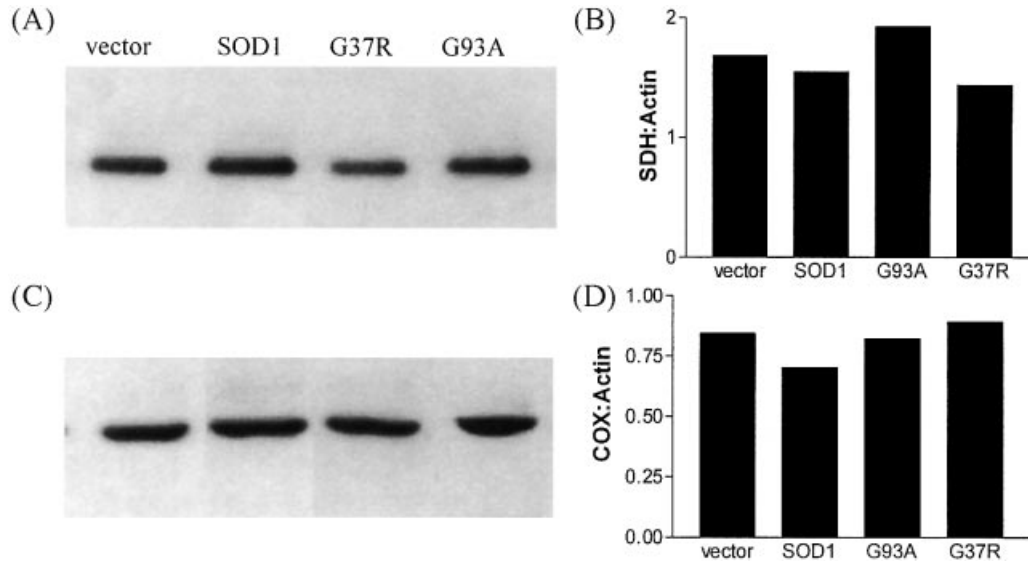
Fig. 3 Protein expression levels of complex II and complex IV in NSC34 cells transfected with vector only, normal human SOD1 or the G37R or G93A mutant forms of human SOD1. Results from one such experiment (representative of three separate experiments) are shown above. (**A**) and (**C**) show Western blots of the iron sulphur centre of complex II and subunit I of complex IV respectively. Densitometric analysis of these blots was carried out and the results expressed as a ratio of actin density following reprobing of the membrane. (**B**) Complex II and (**D**) Complex IV. No significant differences were observed in the expression levels of the complex II and complex IV proteins in any of the cell groups.

cells was seen to result in a significant decrease in activities of both complex II and complex IV compared with control cell lines transfected with vector only or expressing normal human SOD1. The levels of complex I and complex III activity were not significantly altered by the presence of mutant human SOD1 in the cells.

Complex II

Cells expressing G93A mutant SOD1 showed a level of complex II activity of 54% and cells expressing G37R SOD1 showed 56% of the level of complex II activity compared with cells transfected with vector only ($P < 0.01$ in both cases). The activities of complex II in cells expressing mutant SOD1 were also significantly lower than the level observed in cells expressing normal human SOD1 ($P < 0.05$ for both G93A and G37R). The levels of reduction of complex II activity were similar for both of the mutant forms of SOD1. The level of complex II activity was also slightly decreased in the presence of normal human SOD1 at 79% of the level in vector transfected cells ($P < 0.01$), but this decrease was much less than the reduction seen in the presence of the mutant SOD1 protein.

Complex IV

The pattern of decrease seen in complex IV activity in the presence of mutant SOD1 was very similar to that seen with complex II. The presence of normal human SOD1 expression resulted in a decrease in complex IV activity to 73% of that with vector transfected cells ($P < 0.01$). In the presence of

mutant SOD1, however, the activity of complex IV was decreased further, to 56% of the activity of vector transfected cells in G93A cells ($P < 0.001$) and to 50% in G37R cells ($P < 0.001$). The decrease in complex IV activity in mutant cell lines compared with cells expressing normal human SOD1 was also statistically significant in both cases ($P < 0.05$ for G93A and $P < 0.01$ for G37R). There was no difference in the level of alteration of complex IV activity observed between the G93A and G37R cell lines.

Complex I

Complex I activity was not significantly altered in the presence of either normal or mutant human SOD1 ($P = 0.0582$ by one-way ANOVA). Using the Newman–Keuls *post hoc* test as above for other complexes, no significant differences were observed in complex I activities between cells transfected with vector only and those transfected with normal or mutant human SOD1 ($P > 0.05$ in all cases).

Complex III

No differences were noted between cell lines in the activities of complex III ($P = 0.21$).

Presence of mutant SOD1 does not alter levels of complexes II and IV expression in NSC34 cells

The biochemical changes in activities of complexes II and IV were not accompanied by changes in the expression levels of
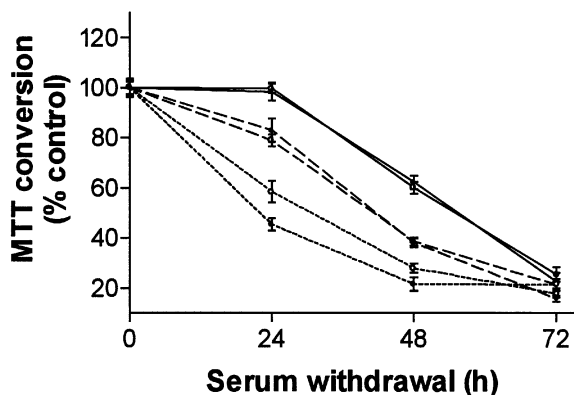
Fig. 4 Cell viability following serum withdrawal was assessed by MTT assay. Viability was analysed in two clonal cell lines for each transfection type. Solid lines represent cells transfected with normal human SOD1, broken lines represent cells transfected with empty vector and dotted lines represent cells expressing G93A mutant SOD1. In each experiment, mean cell viability was calculated from four MTT readings and the data shown represent combined results from three independent experiments for each cell line. The G93A mutant cell lines showed significantly greater cell death compared with untransfected cells ($P < 0.001$; $P < 0.05$ at 24 h and 48 h, respectively following serum withdrawal), whereas the expression of normal SOD1 showed a significant protective effect against cell death ($P < 0.01$; $P < 0.001$ at 24 h and 48 h, respectively following serum withdrawal). Statistical analysis was by ANOVA with Newman–Keuls *post hoc* test.

specific proteins within these complexes, the 30 kDa iron/sulphur subunit of succinate dehydrogenase and subunit 1 of cytochrome *c* oxidase, respectively (Fig. 3). The results shown in Fig. 3 are representative of three separate experiments.

Serum withdrawal results in a decrease in the activities of complexes II and IV of the mitochondrial electron transfer chain in NSC34 cells irrespective of the presence of mutant SOD1

There was no alteration in viability of NSC34 cells expressing mutant human SOD1 under normal culture conditions (data not shown). However, mutant SOD1 was seen to result in an increased cell death when cells were subjected to oxidative stress by serum withdrawal (Fig. 4). This effect has previously been reported in other non-motor neuronal cell lines stably expressing mutant SOD1 (Rabizadeh *et al.*, 1995). We were interested to see what effect the cellular stress of serum withdrawal had on the activities of the mitochondrial respiratory chain complexes in control cells and whether any changes observed were enhanced in the presence of SOD1 mutations. The activities of complexes I–IV were measured under basal conditions and at 6 h, 24 h and 48 h following serum withdrawal in cell lines transfected with vector alone and expressing normal, G93A or G37R mutant SOD1.

In all cell lines, the activities of both complexes II and IV were seen to decrease as the time following serum withdrawal increased (Fig. 5). For complex IV, a decrease in activity was seen in each cell line at 48 h compared with baseline. In vector transfected cells, activity was reduced to 38% of that under basal culture conditions ($P = 0.001$), in normal SOD1 transfected cells to 59% ($P = 0.02$) and to 50 and 48% in G93A and G37R mutant SOD1 cell lines, respectively ($P < 0.0001$ and SOD1 $P = 0.003$). The results for complex II differed slightly: levels of activity at 48 h were decreased in vector transfected cells to 41% of basal activity ($P = 0.01$), to 54% in cells expressing G93A mutant SOD1 ($P = 0.01$), and to 64% in cells expressing G37R mutant SOD1 ($P = 0.03$). The change in cells expressing normal human SOD1, however, was smaller, to 72% of activity in basal conditions and did not reach statistical significance ($P = 0.18$). The activities of complexes I and III showed no significant changes following serum withdrawal. Whilst some decrease was seen in the activity of complex I following 48 h serum withdrawal in the presence of mutant SOD1, these alterations did not reach statistical significance ($P = 0.2152$ for G93A and $P = 0.2671$ for cells expressing G37R mutant SOD1). Indeed, there did not appear to be any additive effect of serum withdrawal and the presence of mutant SOD1 in this cellular model. At 48 h following serum withdrawal, no significant differences were observed in the activities of any of the respiratory chain complexes in vector transfected cell lines compared with mutant cell lines.

Basal energy status of NSC34 cells is not altered in the presence of SOD1 mutations

The functional consequences of the decrease in mitochondrial electron transport on the metabolic energy status of cells expressing mutant SOD1 was further investigated by measuring the ATP : ADP ratio. The ATP : ADP ratios of NSC34 cell lines grown under normal, basal culture conditions were between 1.3 and 2.0 (Fig. 6). ATP : ADP ratios were measured in three independent experiments ($n = 3$); for each experiment, a mean ratio was obtained from three separate measurements. No alteration in the ATP : ADP ratio was seen in the presence of either G93A or G37R mutant SOD1 compared with control cell lines. There were no statistically significant differences in the ATP : ADP ratios measured in the NSC34 cells containing the mutant forms of human SOD1 compared with control cell groups containing vector only or normal human SOD1 (assessed by ANOVA). The results in Fig. 6 represent one clonal cell line for each cell type; these results were replicated in a second set of clonal cell lines.

Cells expressing mutant SOD1 are more sensitive to inhibition of glycolysis

To further assess the bioenergetic significance of abnormalities in the mitochondrial respiratory chain complexes in
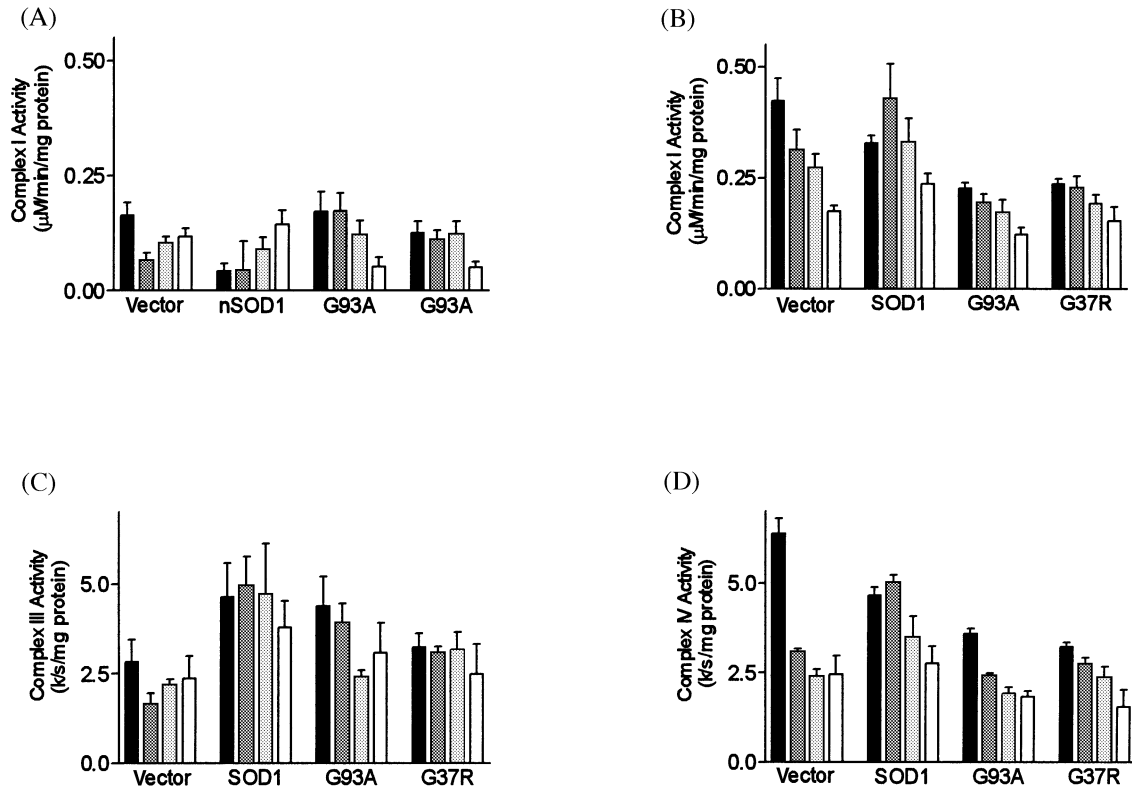
(A)

(B)

(C)

(D)

Fig. 5 Effect of serum withdrawal on mitochondrial electron transfer chain complex activity in control cells transfected with vector only and in cells transfected with normal human SOD1 and with the G93A and G37R mutant forms of human SOD1. The activities of mitochondrial respiratory chain complexes I, II, III and IV were measured under basal culture conditions and at 6 h, 24 h and 48 h following serum withdrawal. Black bars = normal culture conditions (10% serum), dark grey bars = 6 h serum free, pale grey bars = 24 h serum free, white bars = 48 h serum free. (**A**) Complex I activity. (**B**) Complex II activity. (**C**) Complex III activity. (**D**) Complex IV activity. Results are shown as a mean of three independent experiments ($n = 3$); for each experiment, complex activity was measured as the mean of three readings. See text for statistical analysis.
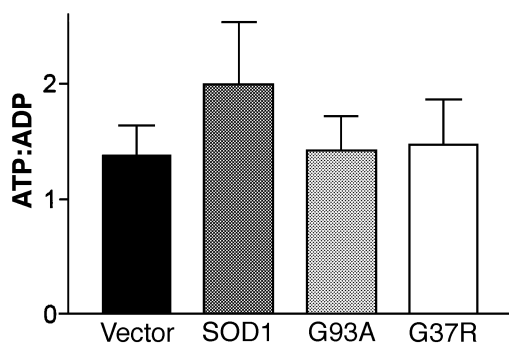

Fig. 6 The ATP : ADP ratio was measured in cells under basal culture conditions. The black bar represents cells transfected with vector only, the dark grey bar represents cells transfected with normal human SOD1 and the light grey and white bars represent cell transfected with G37R or G93A mutant forms of SOD1, respectively. No differences are seen between the basal ATP : ADP ratio in any of the cell lines.
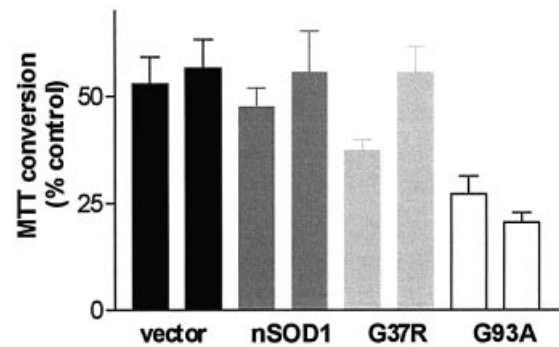

Fig. 7 The effect of inhibition of glycolysis by exposure to iodoacetate (30 μM for 5 h) on cell viability as measured by MTT assay. Data is shown combined from five independent experiments consisting of four observations. Cell death was significantly increased in the presence of mutant SOD1 ($P = 0.0017$ by ANOVA). Results from two clonal cell lines with the same SOD1 phenotype are shown.

mutant cell lines, the effect of inhibiting glycolysis was investigated by the application of iodoacetate (Beltran *et al.*, 2000). The effects of 30 μM iodoacetate were investigated in two clonal cell lines for each cell type and five independent

experiments were carried out, each consisting of four observations (Fig. 7). The addition of iodoacetate exerted a greater toxic effect on mutant cell lines than control cells ($P = 0.0017$ by ANOVA). For example, 30 μM iodoacetate

reduced the viability of cells containing vector only to 58 and 52% in two clonal cell lines, whereas in the presence of G93A mutant SOD1, the viability was reduced to 29 and 23%. The G37R mutation had a less marked effect on cell viability in the presence of iodoacetate. One G37R clone showed a reduction in viability to 37%, whereas the other clone did not produce a consistent detrimental effect (Fig. 7).

Discussion

In this study, we have used a cell line with motor neurone like properties (NSC34), which stably expresses human SOD1 in both wild type and mutant forms. To our knowledge, the data presented here provide the first evidence that the presence of mutant SOD1 within motor neurone like cells causes alterations in the activities of specific components of the mitochondrial respiratory chain. Furthermore, we have also demonstrated for the first time that the expression of mutant SOD1 at levels approximating those seen in human disease results in morphological abnormalities of motor neuronal mitochondria accompanied by functional changes. Under normal culture conditions, the presence of the G93A or the G37R mutant forms of human SOD1 in NSC34 cells leads to a significant decrease in the activities of complexes II and IV. Complexes I and III showed no significant change in the levels of activity. The finding of a decrease in complex IV activity in the presence of mutant SOD1 in this study is supported by other reports which have described decreases in complex IV activity in CNS tissue from sporadic ALS patients. In the spinal cord of sporadic ALS cases, complex IV activity was found to be reduced, most markedly in the motor neurone containing ventral horn (Fujita *et al.*, 1996). A specific decrease in complex IV activity in spinal cord motor neurones of sporadic ALS cases has been previously reported from our laboratory using *in situ* histochemical demonstration of enzyme activity (Borthwick *et al.*, 1999). Complex IV deficiency has also been described in muscle of sporadic ALS patients (Vielhaber *et al.*, 2000), where complex IV negative muscle fibres were found. An out-of-frame mutation in the 5′ end of the mitochondrially encoded subunit 1 of complex IV, which resulted in premature termination of the protein, has also been described in a single patient with a motor neurone disease phenotype (Comi *et al.*, 1998). Other studies have reported findings differing from those reported here, namely increases in the activity of complex I and complex II-III in the frontal cortex, motor cortex, parietal cortex and cerebellum (Bowling *et al.*, 1993; Browne *et al.*, 1998). The apparently contradictory findings of these studies may potentially be explained by the differences in CNS regions studied. The only other study investigating activity specifically in motor neurones also reported a decrease in complex IV activity (Borthwick *et al.*, 1999). The aim of the current study was to provide an experimental model in which some of these discrepancies could be addressed by examining directly the effect of SOD1 mutations on motor neurones, without interference from the presence of other cell types.

It is interesting to note that when the NSC34 cells were subjected to oxidative stress in the form of serum withdrawal, significant decreases were also observed in the activities of complexes II and IV, but not complexes I and III. These decreases were independent of the presence of mutant SOD1 in the cells, i.e. there was no additive effect of the presence of mutant SOD1 and oxidative stress induced by serum withdrawal on the mitochondrial dysfunction observed in the cells. It is interesting that decreases are seen in the same complexes in control cells following serum withdrawal as under basal conditions in the presence of mutant SOD1. This suggests that, under normal culture conditions, cells expressing mutant SOD1 are under higher than normal levels of oxidative stress.

Serum withdrawal induces oxidative stress with the generation of more than one free radical species in the cell. Previous work has shown that immediately upon serum withdrawal, NSC34 cells produce increased levels of both nitric oxide (NO) and superoxide (M.R. Cookson and P. Manning, unpublished data). The relative levels of inhibition produced by different free radical species on the mitochondrial electron transfer chain complexes have been studied. NO has been shown to reversibly inhibit complex IV activity by competing with oxygen for binding sites (Cleeter *et al.*, 1994). Superoxide is thought to most potently inhibit the activity of complexes I and II (Zhang *et al.*, 1990). In the presence of nitric oxide and superoxide, peroxynitrite will be generated. This very reactive free radical species is thought to inhibit predominantly complexes I and II (Radi *et al.*, 1994). The alterations in the respiratory chain complexes II and IV observed in the presence of mutant SOD1 are in keeping with changes produced by these free radical species. We did not detect any changes in the overall levels of expression of representative protein components of these complexes, suggesting that the functional changes observed represent post-translational modifications rather than loss of protein.

We did not observe any changes in the level of activity of complex I in this cell model. As previously demonstrated in other neuronal cells, the basal level of complex I activity in control NSC34 cells is low (Bolanos *et al.*, 1995). In addition, NSC34 cells possess a high level of NADH dehydrogenase activity that is not due to complex I activity (i.e. a component that is not sensitive to inhibition by rotenone). This results in a measurable rate of complex I activity that is very close to the limits of detection. These two factors combined may have affected our ability to detect a decrease in the activity of this complex. All other complexes showed rates that were easily measured and well above the limits of detection.

It is noteworthy that the presence of normal human SOD1 in the NSC34 cells also resulted in some decrease in the activities of complexes II and IV under basal culture conditions, though the changes observed were much less severe compared with those measured in the presence of mutant SOD1. This observation is in keeping with the observation previously reported that over-expression of normal human SOD1 may lead to mitochondrial alterations

in transgenic mice (Jaarsma *et al.*, 2000). Down's syndrome (trisomy 21) patients have a 50% increase in SOD1 protein expression and activity as a consequence of gene dosage excess. The trisomy 16 mouse is a model for Down's syndrome with an equivalent increase in the level of expression of normal murine SOD1. Interestingly, mitochondrial dysfunction (Schuchmann *et al.*, 1998) and abnormally shaped mitochondria within neurones (Bersu *et al.*, 1998) have been demonstrated in these mice. When NSC34 cells were subjected to serum withdrawal in the presence of normal human SOD1, there was no reduction noted in complex II, which differed from the findings in the other cell groups. This may be relevant to the observation that the presence of normal human SOD1 in this cell model exerts a protective effect against oxidative stress induced by serum withdrawal.

Despite the decrease in complexes II and IV activities observed, no alteration was seen in the ATP : ADP ratio of cells expressing mutant SOD1 and grown under basal culture conditions. It has previously been demonstrated that cells grown under basal culture conditions may be able to maintain their ATP : ADP ratio despite impairment of mitochondrial electron transfer chain activity (James *et al.*, 1999). NSC34 cells are grown in medium containing high levels of glucose and it is therefore possible that they are able to maintain their ATP : ADP ratio at adequate levels via the glycolytic pathway. It may therefore be possible to demonstrate a defect in basal cellular energy status in cells cultured in the presence of lower levels of glucose. The observation of a lack of alteration of ATP : ADP ratio may be relevant to the fact that no decrease in viability of the motor neuronal cells containing mutant human SOD1 was observed under basal culture conditions.

It is possible for cells grown in culture to rely on glycolysis as their major source of ATP production rather than oxidative phosphorylation. In order to investigate the possibility that the ability of NSC34 cells to produce ATP by glycolysis masked any effects of a loss of function of the mitochondrial electron transfer chain, we investigated the effect of inhibiting glycolysis. Cell lines expressing mutant human SOD1 did show an increased level of cell death following inhibition of glycolysis by iodoacetate. These data further confirm findings that there is a defect in oxidative phosphorylation in NSC34 cells expressing mutant SOD1.

Until recently, SOD1 has generally been considered to be expressed primarily in the cytosolic compartment of cells, whereas Mn SOD (SOD2) is expressed in the mitochondria (Pardo *et al.*, 1995; Shaw *et al.*, 1997). This subcellular localization pattern of the two intracellular SOD enzymes has raised questions as to how mutations in SOD1 might cause mitochondrial damage. Recently, however, a role for SOD1 in the mitochondria and its presence in the intermembrane space of the mitochondria of yeast has been demonstrated (Sturtz *et al.*, 2001). A previous study showing localization of SOD1 in the mitochondria (Weisiger and Fridovich, 1973) was questioned, due to the possibility of contamination of the mitochondrial preparation with cytosolic protein (Geller and

Winge, 1982). However, it now appears that this observation was correct. It has been suggested that the role of SOD1 in the intermembrane space is the removal of superoxide released into this space by the ubisemiquinone anion (Sturtz *et al.*, 2001). Mn SOD is localized in the mitochondrial matrix where it could not fulfil this role. The mitochondrial fraction of SOD1 may therefore be important for the cell specific toxic effects generated by the expression of the mutant protein.

In conclusion, the data presented from this cell culture model of SOD1 related FALS suggest that one important mechanism by which mutant SOD1 causes motor neurone injury involves inhibition of specific components of the mitochondrial electron transfer chain. Biochemical dysfunction is accompanied by morphological changes in mitochondria and functional changes in cellular energy metabolism demonstrated by a reduced ability of cells to withstand inhibition of glycolysis under basal culture conditions. The observation that the same alterations in mitochondrial function are seen in cells expressing mutant SOD1 as following an imposed general oxidative stress in control cells supports the hypothesis that the toxic gain of function exerted by mutant SOD1 involves altered intracellular free radical handling. The precise mechanism through which the presence of mutant SOD1 leads to the mitochondrial effects reported in this study is an interesting area for further work. It has been demonstrated that motor neurones *in vitro* are particularly vulnerable to cell death in the face of chronic inhibition of complexes II and IV of the mitochondrial respiratory chain, compared with other neuronal groups (Kaal *et al.*, 2000). Further work is necessary to elucidate the molecular basis of this vulnerability. The mitochondrial alterations demonstrated in this cell culture model of SOD1 related FALS suggest that therapeutic measures targeted to protection of mitochondrial respiratory chain function may be useful in this subgroup of ALS patients.

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