

Progress report of the previous ARS grant (in English only)

A full progress report must be provided 6 months after the beginning of the execution of the contracts of 1 year length (or 12 months for contracts of 2 years length), this is valid for all the contracts mentioned on page 5, except those which were already reported on in a previous application. Please begin the description with the page provided and continue for a maximum of 5 pages.

Summary of the initial objectives

As protein synthesis occurs in the cell body, anterograde axonal transport (to the nerve ending) along the cytoskeleton array is essential to supply axons with newly synthesized proteins. Retrograde axonal transport (to the motor neuron cell body) is also essential for motor neuron survival as it controls neurotrophic factor signals and vesicle recycling. Due to the length of their axon, motor neurons are particularly susceptible to any axonal transport defect. Defects in axonal transport can cause motor neuron diseases as shown by the discovery of gene mutations/dysfunctions for components of the axonal transport in subsets of both mouse models and human cases. Several lines of evidence have shown that axonal transport machinery is impaired early in life in mSOD1 mice. These observations emphasise that axonal protein content needs to be further evaluated during the course of ALS disease.

The aim of this study was to obtain a comprehensive overview of the axonal protein content during the course of ALS disease in mSOD1 mice. The use of an animal model makes it possible to investigate different stages of the disease progression including the presymptomatic stage. Two dimensional gel electrophoresis have been used to analyze the differences of axonal proteome between mice overexpressing mutant (mSOD1), wild type human SOD1 (hSOD1) and non transgenic mice at 30 (prior to neuronal defect), 60 (onset of distal denervation in muscles), 90 (loss of ventral root axons) and 120 (loss of motor neuron cell bodies) days old. We planned to compare 1) total protein content and 2) phosphoprotein content of the sciatic nerve of mouse groups after 2D gel electrophoresis.

Main results obtained

The right and left sciatic nerve have been dissected from G93A transgenic mice of 30, 60, 90 and 120 days

old, according to the temporal quantitative pathological analysis performed in these mice (Fischer et al., 2004). Age- and gender-matched wild type (WT) mice (that do not express any form of hSOD1) and mice overexpressing non mutated human SOD1 (hSOD1) have been used as controls. All animal procedures have received approbation of the competent French government authorities.

A total of 12 animals per group have been used in this study.

-6 were used for two-dimensional gel electrophoresis (sciatic nerve)
-3 for western blot analysis (sciatic nerve, hypoglossal nerve)
-3 for immunofluorescence staining (sciatic nerve, hypoglossal nerve and ventral and dorsal roots)

Sciatic nerve protein extracts have been prepared in lysis buffer containing proteinase/phosphatase inhibitors. Aliquots of 500 µg of protein will have been precipitated by standard trichloroacetic acid/acetone procedure. Two-dimensional gel electrophoreses have been run. Gels have been stained by a fluorescent stain procedure and digitalized with an Ettan™ DIGE Imager camera. Image analysis has been done by the ImageMaster 2D Platinum v6.0 software. Variation of protein spot volumes has been calculated from 6 animals per group and compared using statistical analysis. Six hundred spots have been analyzed for each time point. Selected spots (significant 1.3 fold variation, power > 80%) have been excised and analyzed by mass spectrometry (MALDI-TOF) and confirmed by LC-MS/MS. Some of the proteins differentially represented have already been identified.

Position of the spots differentially represented in 30 day-old G93A in 120 day-old G93A

Few differences are noted in the sciatic nerve at 30 day-old between G93A, WT and hSOD1 groups. Only 4 proteins presented modified levels in G93A and hSOD1 mice sciatic nerve compared to WT. Three are decreased and one is increased. Identification of these proteins is in progress. However observed modifications resulted from overexpression of human (mutated or normal) SOD1 in mice and are not related to the disease.

In contrast, G93A group is different from both hSOD1 and WT group at 120 day-old. Level of 9 spots is increased (Table 1) whereas level of 23 spots is decreased (Table 2).

Among these proteins, a majority of them are cytoskeletal proteins. The decrease in neurofilament proteins and the consecutive increase in peripherin and vimentin levels were expected and have already been reported in ALS mouse models (Beaulieu et al. 1999; Perrin et al., 2005). Some proteins such as beta enolase and creatine kinase M-type are muscle specific isoforms. Their detection reflects that muscle fragments probably contaminated the sciatic nerve tissue. Several enzymes (Pyruvate kinase isoenzymes M1/M2, triose phosphate isomerase and ATP synthase subunit beta) are essential for correct energy production. Two proteins (ubiquitin carboxyl-terminal hydrolase and DJ-1) are involved in ubiquitin pathway.

Two isoforms of alpha-1-anti-trypsin are strongly increased in the sciatic nerve of 120 day-old G93A mice. This serpin (serine protease-like protein) has been found in neurofilament aggregates in ALS patient spinal cord (Chou et al. 1998). Its increase is thus surprising in view of the concomitant neurofilament decrease observed here. An increase in this protein has also been reported in cerebrospinal fluid of ALS patients (Brettschneider et al. 2008) and of patients with various neurological disorders such as multiple sclerosis (Dumont et al. 2004; Hammack et al. 2004; Lehmsiek et al. 2007a), frontotemporal dementia (Hansson et al. 2004) and Guillain-Barré syndrome (Lehmsiek et al. 2007b). Whether the increase we observed occurred within the motor axons remains to be determined.

Rank	Anova (p)	Fold q	Value	Power	spot ID	Notes
3	0,00129797	3,4	0,03335159	0,970072	ID: 0508	Peripherin
9	0,00082677	2,7	0,02868521	0,98248108	ID: 0522	ATP synthase subunit beta // vimentin
10	8,3298E-05	2,7	0,01193772	1	eID: 0005	Alpha-1-antitrypsin 1-6 precursor
13	0,00030067	2,5	0,02322725	0,99569207	eID: 0007	Alpha-1-antitrypsin 1-6 precursor
17	0,00125893	2,3	0,03335159	0,97109096	eID: 0009	Identification in progress
21	0,00021363	2,2	0,02225364	0,99749794	ID: 0360	Identification in progress
30	0,00787559	1,9	0,08750903	0,84076116	ID: 0841	Identification in progress
61	0,00373213	1,6	0,06239618	0,91332118	eID: 0035	Creatine kinase B-type
64	0,0020046	1,5	0,04315578	0,95218704	ID: 0985	Peroxiredoxin-1

Table 1: Proteins which are increased in the sciatic nerve of 120 day-old G93A mice

Rank	Anova (p)	Fold q	Value	Power	spot ID	Notes
1	0,00089795	-6,1	0,02868521	0,98060043	eID: 0098	Neurofilament light polypeptide // Neurofilament medium polypeptide
6	0,00369136	-2,9	0,06239618	0,9141655	eID: 0097	Neurofilament light polypeptide
8	0,00070249	-2,9	0,02634397	0,98574903	eID: 0100	Neurofilament light polypeptide
11	0,00759784	-2,7	0,08750903	0,84496458	ID: 0565	Beta enolase
12	0,00208936	-2,6	0,04352953	0,95012025	ID: 0640	Creatine kinase M-type
14	0,00767604	-2,5	0,08750903	0,84377331	ID: 0647	
19	8,59E-05	-2,3	0,01193772	1	ID: 0862	
22	4,97E-05	-2,1	0,01193772	1	eID: 0101	
23	0,00503428	-2,1	0,06998189	0,88791179	ID: 0344	
34	0,00046208	-1,9	0,02322725	0,99189567	ID: 0536	Neurofilament medium polypeptide
37	0,00793308	-1,8	0,08814784	0,83990055	ID: 0165	
38	0,00051879	-1,8	0,02322725	0,99048056	eID: 0102	Neurofilament light polypeptide
50	0,00046671	-1,7	0,02322725	0,99178136	ID: 0535	Neurofilament medium polypeptide
51	0,00374366	-1,7	0,06239618	0,91308295	ID: 0942	Triose phosphate isomerase
52	0,01067538	-1,7	0,10680253	0,80215722	eID: 0033	Neurofilament light polypeptide
55	0,00479605	-1,7	0,06998189	0,89234991	ID: 0993	Peroxiredoxin-1
60	0,00280617	-1,6	0,05456592	0,93332694	ID: 0850	
65	0,00503855	-1,5	0,06998189	0,88783298	ID: 0938	
72	0,009831	-1,4	0,10240921	0,81314376	ID: 0439	Pyruvate kinase isozymes M1/M2
80	0,0065877	-1,4	0,0869235	0,86093469	ID: 0732	
86	0,0072869	-1,4	0,08750903	0,84976318	ID: 0520	Neurofilament light polypeptide
88	0,00460285	-1,4	0,06998189	0,89601469	ID: 0968	Protein DJ-1 // Ciliary neurotrophic factor
95	0,02697576	-1,3	0,13245677	0,65288771	ID: 0929	Ubiquitin carboxyl-terminal hydrolase isozyme L1
100	0,02142515	-1,3	0,12019179	0,69410172	eID: 0103	Ubiquitin carboxyl-terminal hydrolase isozyme L1

Table 2: Proteins which are decreased in the sciatic nerve of 120 day-old G93A mice

We also performed a time course analysis of the modified proteins during the course of the disease in ALS mice. Examples of this analysis are presented below for:

-two proteins upregulated during the course of the disease (alpha-1-antitrypsine 1-6 precursor and vimentin)

-two proteins downregulated during the course of the disease (NF-L and pyruvate kinase M1/M2)

-DJ1 which is strongly decreased in at one particular time point: 90 day-old

-Peroxiredoxin which is particularly increased in 60 day-old sciatic nerve

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Alpha-1-antitrypsine 1-6 precursor vimentin

Neurofilament light Pyruvate kinase isoenzymes M1/M2

DJ-1 Peroxiredoxin-1

Identification of the remaining differentially expressed proteins is in progress. Western blot will be done to confirm the temporal changes of protein levels of various protein groups we have identified. Immunofluorescence followed by confocal analysis will also be performed to determine whether these modifications occurred in the motor or the sensitive axonal compartment.

Problems encountered and solutions adopted

Problems we encountered were linked to:

- a poor reproduction rate of the G93A mouse line which led to a long time to collect the tissues.
 - difficulties to find appropriate conditions allowing efficient axonal proteins solubilization compatible with 2D gel electrophoresis analysis.
 - failure to isolate axonal phosphoproteins (using several commercially available phosphoprotein enrichment kits) at a level sufficient for subsequent 2D gel electrophoresis analysis.
- Perspectives brought by results of the project

This study will provide a list of axonal proteins that may help to understand how mSOD1 causes axonal transport defect and neuronal death.

Thanks to our collaborative work with Dr. Danielle Seilhean (neuropathologist, Laboratory of neuropathology), we will also study the level of these proteins in nerve fragments from post-mortem ALS tissues.

The genes encoding the proteins with a modified expression profile will become new candidates for genetic screening. Interestingly, one of the temporally modified proteins we have identified is coded by the DJ-1 gene. Homozygous mutations in this gene were found in a family presenting a complex disorder of early onset parkinsonism-dementia-ALS (Annesi et al., 2005). This result validates our proteomic approach to find candidate genes associated with ALS phenotype. Such a proteomic approach has already helped identify the causing gene in disease for which the traditional genetic approaches were limited by small family sizes (Schessl et al., 2008).

Results from our study will be published as soon as possible.

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