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RESEARCH PAPER

Misfolded SOD1 inclusions in patients with mutations in *C9orf72* and other ALS/FTD-associated genesKarin Forsberg,^{1,2} Karin Graffmo,¹ Bente Pakkenberg,³ Markus Weber,⁴ Martin Nielsen,⁵ Stefan Marklund,¹ Thomas Brännström,¹ Peter Munch Andersen^{6,2}

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ABSTRACT

Objective A hallmark of amyotrophic lateral sclerosis (ALS) caused by mutations in *superoxide dismutase-1* (*SOD1*) are inclusions containing SOD1 in motor neurons. Here, we searched for SOD1-positive inclusions in 29 patients carrying ALS-linked mutations in six other genes.

Methods A panel of antibodies that specifically recognise misfolded SOD1 species were used for immunohistochemical investigations of autopsy tissue.

Results The 18 patients with hexanucleotide-repeat-expansions in *C9orf72* had inclusions of misfolded wild type (WT) SOD1^{WT} in spinal motor neurons. Similar inclusions were occasionally observed in medulla oblongata and in the motor cortex and frontal lobe. Patients with mutations in *FUS*, *KIF5A*, *NEK1*, *ALSIN* or *VAPB*, carried similar SOD1^{WT} inclusions. Minute amounts of misSOD1^{WT} inclusions were detected in 2 of 20 patients deceased from non-neurological causes and in 4 of 10 patients with other neurodegenerative diseases. Comparison was made with 17 patients with 9 different *SOD1* mutations. Morphologically, the inclusions in patients with mutations in *C9orf72HRE*, *FUS*, *KIF5A*, *NEK1*, *VAPB* and *ALSIN* resembled inclusions in patients carrying the wildtype-like *SOD1*^{D90A} mutation, whereas patients carrying unstable *SOD1* mutations (A4V, V5M, D76Y, D83G, D101G, G114A, G127X, L144F) had larger skein-like SOD1-positive inclusions.

Conclusions and relevance Abundant inclusions containing misfolded SOD1^{WT} are found in spinal and cortical motor neurons in patients carrying mutations in six ALS-causing genes other than *SOD1*. This suggests that misfolding of SOD1^{WT} can be part of a common downstream event that may be pathogenic. The new anti-SOD1 therapeutics in development may have applications for a broader range of patients.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative syndrome characterised by adult-onset progressive loss of motor neurons with a focal onset of progressive paresis and muscle wasting.^{1–3}

Some 5%–10% of patients with ALS self-report a familial predisposition (fALS), and the remaining cases are denoted as sporadic ALS (sALS). Since 1993, mutations in 37 genes have been reported to predispose to ALS. In Caucasians, the most commonly reported mutation is a GGGGCC repeat-expansion in *C9orf72* (*C9orf72HRE*), found in 8%–10% of patients with ALS. Missense, substitution or deletion mutations in the genes encoding superoxide dismutase-1 (*SOD1*), TAR-DNA-binding protein 43

(*TDP-43*), fused in sarcoma (*FUS*) and kinesin heavy chain isoform 5A (*KIF5A*) are found in 2%–6% of European patients. The remaining gene mutations are rare. ALS is both genetically and clinically heterogeneous and the reported predisposing genes pleiotropic.⁴ Neuropsychological examinations and imaging studies can show involvement of the frontotemporal lobes,^{5,6} though this may remain subclinical.⁷ In particular, carriers of *C9orf72HRE*, *FUS*, *VCP* and *TBK1* mutations may develop frontotemporal dementia (FTD) sometimes without overt ALS. Unfortunately, there have been few comparative neuropathological examinations in patients with different gene defects.

The first ALS-causing gene discovered was *SOD1*⁸ and since 1993 more than 210 mutations have been found (<http://alsod.iop.kcl.ac.uk/>).⁹ While some of the mutant SOD1s (eg, C6S, D90A, E100K, L117V) are sufficiently stable and active to yield normal SOD1 activity in human tissues,¹⁰ others, for example, A4V, V5M, D76Y, D83G, G127Gfs*5 (alias ‘G127X’) are unstable, are rapidly targeted for degradation and can only be detected in minute amounts.^{11,12}

Neuronal inclusions containing aggregated SOD1 are recognised pathological hallmarks of ALS caused by *SOD1* mutations in patients and in transgenic (Tg) animal models overexpressing mutant human (h) SOD1s.^{11,13} In the Tg models, two structurally different strains have been found.¹⁴ When inoculated into the lumbar spinal cord of adult mice, both strains induce cell-to-cell propagating templated hSOD1 aggregation primarily in the motor neurons resulting in rapidly progressing ALS-like fatal diseases.¹⁵ Similar effects have been obtained inoculating SOD1-aggregates purified from the spinal cord of a patient with the *SOD1*^{G127X} mutation.¹⁶ This prion-like action could be the primary pathogenic mechanism of SOD1-provoked ALS, which prompts exploration of SOD1 in ALS caused by other genes.

Previously, we examined spinal cord tissue from 37 patients with sALS and found numerous inclusions containing misfolded wild-type SOD1 (misSOD1^{WT}) in the cytoplasm and nuclei of neurons and glial cells.^{17,18} The presence of misSOD1^{WT} in motor neurons of patients with ALS was later observed by other groups with the differences that antibodies against other SOD1-epitopes were used and inclusions were not found in all patients studied.^{19–22} Supporting that misSOD1^{WT} may cause neurodegeneration, expression of hSOD1^{WT} in Tg



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Neurodegeneration

mice result in a fatal ALS-like disease with neuronal inclusions containing human misSOD1 aggregates.²³ Also, in vitro studies in which motor neurons are cocultured with astrocytes derived from patients with ALS suggests an involvement of misSOD1^{WT} in the pathogenesis.²⁴ Collectively, there is emerging evidence that misSOD1^{WT} is neurotoxic and participate in ALS pathogenesis in general and not only in patients carrying mutations in *SOD1*.

In the present study, we investigated whether inclusions containing misSOD1^{WT} are present in patients carrying mutations in six other ALS-causing genes.

MATERIALS AND METHODS

Human subjects

Since 1993, tissue has been collected at autopsy at Umeå University Hospital, Sweden from patients with ALS and FTD. The EFNS Criteria for Managing ALS²⁵ and the Neary Criteria for FTD²⁶ were used to set the diagnosis. Blood leucocyte DNA from autopsied patients (n=97) was analysed for a panel of ALS-linked genes.^{12 27 28} Of these patients, 18 had *C9orf72HRE* (confirmed by Southern blot in CNS tissue),²⁹ 2 had *FUS* mutations, 1 an *ALSIN* mutation (censored, manuscript in preparation), 1 a *VAPB* mutation, 1 a *NEK1* mutation⁶ and 11 had *SOD1* mutations. Eleven patients carried the coding rs113247976 SNP (P986L) in *KIF5A* recently found to predispose to ALS, three of these being double mutants *C9orf72HRE* plus rs113247976 and one A4V *SOD1* plus rs113247976 SNP.²⁸

Six additional patients with *SOD1* mutations were obtained from Danish and Swiss pathology units. Similar tissues were obtained from 10 control patients with other neurodegenerative conditions and from 20 patients without neurological diseases (table 1, online supplementary table 1).

All procedures were performed in accordance with the 1964-Helsinki Declaration with amendments and were approved by the Ethical Review Boards for Medical Research at the relevant institutions in Sweden, Denmark and Switzerland. Informed consent for performing autopsies for research purposes was obtained from the patients and/or the next of kin.

Tissue preparation and staining for misSOD1

Tissue samples from the spinal cord (cervical, thoracic and lumbar regions), medulla oblongata, motor cortex, frontal lobe and cerebellum were collected and fixed by immersion in 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. After fixation, tissues were embedded in paraffin, sectioned (4 µm), and stained with H&E. The antigen retrieval procedure is essential for detecting misSOD1 and should be mild, since heavily denaturing protocols may cause misfolding of the abundant natively folded SOD1. A well-functional retrieval treatment for sections is 10 min in preheated citrate buffer, pH 6.3, without boiling.¹⁸

Immunohistochemical staining (IHC) was initially performed using the ES system and ES reagents and, from 2014, by the more sensitive BenchMark XT automated staining system (both from Ventana Medical System, Illkirch, France) and aminoethylcarbazole or Fast Red was used for development. For immunofluorescent studies, a corresponding fluorescent-labelled secondary antibody was used. Sudan Black diluted to 0.3% was used to avoid autofluorescence. The anti-SOD1 antibodies were diluted to a final concentration of 0.962 µg/mL for 131–153 Ra-ab, 3.7 µg/mL for 57–72 Ra-ab and 1.612 µg/mL for G127X Ra-ab. The sections were examined using a BX53 light microscope (Olympus) and by confocal laser microscopy using a Zeiss LSM

Table 1 Summary of clinical features of the 46 patients with ALS/FTD

Case	Mutated gene	Diagnosis	a.o.	First symptom	d.d.	c.i.	Sex	p.m.
1	C9orf72HRE	ALS	64	Hand	25	No	M	1.5
2	C9orf72HRE	fFTD	60	Disinhibition	168	Yes	M	1
3	C9orf72HRE	fALS	59	Hand	36	No	M	4
4	C9orf72HRE	fALS-FTD	51	Disinhibition	43	Yes	M	2.5
5	C9orf72HRE	sPBP	69	Bulbar	21	No	M	3
6	C9orf72HRE	ALS	63	Leg	32	No	M	1.5
7	C9orf72HRE	fALS	58	Leg	18	No	M	3
8	C9orf72HRE	fALS	49	Arm	36	No	M	1
9	C9orf72HRE	fALS	51	Arm	130	No	M	2
10	C9orf72HRE	fALS	66	Arm	29	No	M	2
11	C9orf72HRE	PBP+FTD	76	Bulbar	60	Yes	F	1.5
12	C9orf72HRE	fALS	53	Leg	28	No	F	0.5
13	C9orf72HRE	sPBP	72	Bulbar	35	Yes	F	1
14	C9orf72HRE	fALS +FTD	67	Leg	35	Yes	M	1.5
15	C9orf72HRE	fALS	54	Arm	12	No	F	2
16	C9orf72HRE+KIF5A	fFTD	60	Disinhibition	133	Yes	M	3
17	C9orf72HRE+KIF5A	fALS	63	Leg	30	No	F	2
18	C9orf72HRE+KIF5A	ALS	44	Leg	65	n.a.	M	0.5
19	KIF5A	ALS	61	Arm	13	No	F	1.5
20	KIF5A homozygous	sALS	61	Leg	61	No	F	1
21	KIF5A	PBP	67	Bulbar	27	No	F	1
22	KIF5A	ALS	67	Thoracic	17	No	M	1
23	KIF5A	PMA	68	Arm	63	n.a.	F	1.5
24	KIF5A	PBP	65	Bulbar	13	No	M	1.5
25	NEK1 R812X	fALS	75	Hand	38	Slight	F	1.1
26	VAPB S160delS	sPBP	55	Bulbar	43	Yes	M	0.5
27	FUS R495X	fPBP	16	Bulbar	10	No	F	4
28	FUS Q23L	sPBP	35	Bulbar	175	Yes	M	2.5
29	ALSIN+KIF5A	sALS	50	Legs	224	Yes	M	2
30	SOD1 V5M	fALS	71	Leg	40	No	M	2.2
31	SOD1 A4V+KIF5A	fALS	73	Arm	10	Yes	F	1.9
32	SOD1 G127X	fALS	72	Thoracic	24	No	M	1.1
33	SOD1 G127X	fALS	53	Thoracic	6	No	M	1.5
34	SOD1 G127X	fALS	59	Arm	32	No	F	n.a.
35	SOD1 G114A	fALS	38	Arm	36	No	M	1.5
36	SOD1 G114A	fALS	29	Leg	14	Yes	F	4
37	SOD1 D76Y	fALS	49	Leg	204	No	M	n.a.
38	SOD1 D83G	fALS	49	Leg	37	No	F	2
39	SOD1 D101G	fPMA	48	Arm	6	n.a.	M	n.a.
40	SOD1 L144F	fALS	71	Hand	65	No	M	0.2
41	SOD1 D90A	fALS	31	Leg	265	No	M	2
42	SOD1 D90A	fALS	58	Leg	101	No	M	2
43	SOD1 D90A	fALS	48	Leg	316	No	M	1.5
44	SOD1 D90A	fALS	53	Leg	131	No	F	2.5
45	SOD1 D90A	fALS	56	Leg	174	No	M	1.5
46	SOD1 D90A	fALS	38	Leg	60	No	F	2

Cases nr 20, 29 and 41–46 were homozygous, all others heterozygous for the respective genes. ALS, amyotrophic lateral sclerosis; a.o., age at onset (years); c.i., cognitive impairment; d.d., disease duration (months); fFTD, familial frontotemporal dementia; F/M, female/male; p.m., postmortem time (days).

710 confocal microscope and were analysed using the Zen 2011 SP7 software. The presence of intracellular misSOD1 inclusions was rated by two independent examiners according to a 4-point semiquantitative scale.¹⁸

RESULTS

Antibodies specifically detect misfolded SOD1

SOD1 is abundant in the CNS constituting 0.1% of the tissue protein.^{11 17} Carriers of *SOD1* mutations show inclusions containing misfolded aggregated SOD1. It is estimated that ≈1% of the total SOD1 protein is contained in such inclusions.^{11 30}

Any misSOD1 inclusions occurring in patients with ALS who lack mutations in *SOD1* are likely to be equally scarce. Thus, to detect such species, antibodies that lack reactivity with natively folded SOD1 must be used. Use of Rabbit 1, a Ra-ab raised against native SOD1 with native/denatured reactivity ratio of 0.7/1,³¹ is found to stain tissues heavily obscuring detection of inclusions containing misSOD1 (online supplementary figure 1C-D). Moreover, the handling of tissue specimen and antigen retrievals have to be lenient to prevent misfolding of native SOD1. We have developed a panel of antibodies, raised against peptides in the human SOD1^{WT} sequence, which only react with disordered or misSOD1 species but show no reactivity against natively folded human SOD1. They were highly specific for SOD1 in western blots of human CNS tissue extracts.^{17 18} The two primary antibodies used here were rabbit anti-human antibodies (Ra-ab) to misfolded SOD1, raised against peptides corresponding to amino acids (aa) 57–72 and aa131–153. The 57–72 Ra-ab and 131–153 Ra-ab were chosen because they are well characterised^{17 18} and detect misfolded SOD1 in human tissue studies equally well. For detection of mutated SOD1 in patients with the *SOD1* truncating mutation SOD1^{G127X}, we developed a mutant-specific peptide antibody, G127X Ra-ab, directed against the neopeptide sequence unique to this mutation (aa 123–127GQRWK-stop). This antibody reacts exclusively with the mutant protein.¹¹ To test the specificity for misSOD1 in histopathological studies, the 131–153 Ra-ab was preincubated with the immunising peptide in increasing concentrations. This resulted in gradual and finally full disappearance of immunoreactivity (online supplementary figure 1E-H).

Inclusions containing misSOD1^{WT} can be seen in patients carrying *C9orf72HRE*

Different regions of the brain and spinal cord from 18 patients carrying *C9orf72HRE* were stained for misSOD1 and analysed. We found small dot-like granular inclusions of misSOD1^{WT} in spinal cord motor neurons in all 18 patients (tables 2 and 3), irrespective of whether the clinical diagnosis was ALS, FTD or both. The inclusions measured 0.5–3 µm were scattered in the cytoplasm of motor neurons and were particularly abundant in the soma (figure 1A,B,D and figure 2F–H) but also present in axons (figure 1A,B). The proportion of spinal motor neurons carrying misSOD1^{WT} inclusions varied between patients; usually, only a few motor neurons per section carried misSOD1^{WT} inclusions. However, in some patients, the proportion was higher. Inclusions were found at all levels of the spinal cord (cervical, thoracic and lumbar) in most patients (table 3). MisSOD1^{WT} inclusions were also present in the neurons of Clark's nuclei. Furthermore, small inclusions of misSOD1^{WT} were found in the hypoglossus (figure 3D), vagus, facialis and accessorius nuclei. The inferior olivary nucleus did not show misSOD1^{WT} inclusions and was normal in most patients.

In the motor cortex, degenerated neurons containing numerous small misSOD1^{WT} inclusions were detected in 7 of 12 *C9orf72HRE* patients investigated, similar in size to the ones seen in spinal motor neurons (figure 3B,C and table 2) and located both in the cytoplasm and the nucleus. The proportion of motor cortex neurons that carried misSOD1^{WT} inclusions was much smaller than the proportion of spinal motor neurons. In the frontal cortex, 11 out of 16 patients with *C9orf72HRE* carried misSOD1^{WT} inclusions. Noticeably, in some patients, senile plaques were seen in the motor and frontal cortex, these stained heavily for misSOD1^{WT}.

In the cerebellum of all 12 patients investigated, loss of Purkinje cells and degeneration of white matter was observed. Staining with anti-misSOD1 antibodies revealed misSOD1^{WT}-stained glial cells surrounding either degenerated Purkinje cells or empty baskets. Staining was also found in the neuropil in both grey and white matter as well as in glial cells in the same regions (online supplementary figure 1A, B). In the granular and molecular layers of the cerebellum, p62-positive neuronal cytoplasmic inclusions were observed, a finding proposed to be pathognomonic for this subgroup of ALS.³² No misSOD1^{WT} staining was seen in the granular layer in the patients with *C9orf72HRE*.

Two of the patients with *C9orf72HRE* were siblings, one with FTD and the other with ALS (patients 2 and 3, respectively, table 2). The sibling with ALS had a significant loss of spinal motor neurons, whereas the sibling with FTD appeared to have a normal number of spinal motor neurons. However, both had misSOD1^{WT} inclusions of similar morphology in motor neurons at all levels of the spinal cord. No misSOD1^{WT} inclusions were detected in the medulla oblongata. Comparing their frontal cortices, the sibling with FTD had many extracellular senile plaques which stained heavily for misSOD1^{WT} and amyloid-β. This was not seen to the same extent in the ALS sibling. In both, misSOD1^{WT} was rarely observed in cortical glial cell nuclei (table 2).

Patients with *FUS*, *NEK1*, *KIF5A*, *VAPB* and *ALSIN* mutations

We found granular inclusions of misSOD1^{WT} in motor neurons of the spinal cord in all patients with *FUS*, *NEK1*, *KIF5A*, *VAPB* or *ALSIN* mutations (figures 1C,E–G and 2I and table 2). The misSOD1^{WT}-positive inclusions were morphologically similar to the ones observed in patients with *C9orf72HRE* and in patients with apparently sporadic ALS.¹⁷ Moreover, misSOD1^{WT} pathology in the patients with *FUS*, *NEK1*, *KIF5A*, *VAPB* or *ALSIN* mutations included all levels of the spinal cord and brainstem motor nuclei (figure 3E,G), areas of the motor cortex (figure 3A), frontal lobe and the cerebellum. In the patients with *FUS* mutations, large cytoplasmic FUS-positive inclusions were found in the spinal cord and medulla oblongata when stained with an anti-FUS antibody. In the cerebellum, the same type of histopathology was observed as in the patients with *C9orf72HRE*, which included staining of misSOD1^{WT} in glial cells of the molecular and granular layers surrounding degenerated Purkinje cells. The patients with *VAPB* and *ALSIN* mutations in particular had these changes, whereas the cerebellum in the patients with *FUS* mutations was less affected. We conclude that all studied patients with *FUS*, *NEK1*, *KIF5A*, *VAPB* and *ALSIN* mutations had misSOD1^{WT} inclusions in the spinal cord and brainstem motor nuclei and occasionally also in the motor cortex and frontal lobe.

Comparison with patients carrying different *SOD1* mutations

For comparison, we stained tissue from 17 patients carrying *SOD1* mutations (tables 2 and 3). The six patients homozygous for the *SOD1*^{D90A} mutation had small granular inclusions (figure 1I) morphologically similar to the inclusions described above in patients carrying mutations in *C9orf72HRE*, *FUS*, *NEK1*, *KIF5A*, *ALSIN* and *VAPB* and previously in sALS.^{17–19 22} This could be expected since the *SOD1*^{D90A} mutation produces an essentially fully stable SOD1^{D90A} protein with biophysical properties of SOD1^{WT}.³³ All patients with the *SOD1*^{D90A} mutations had mild cortical atrophy with neuronal loss, reactive gliosis and microvacuolisation of the three superficial cortical laminae. MisSOD1^{D90A} inclusions in the motor cortex

Table 2 Immunohistochemical results of staining for misfolded SOD1 using the misSOD1 peptide antibodies: 131–153 Ra-ab (cases #1–31, #35–56) and G127X Ra-ab (cases #32–34)

Case	Mutated gene	Spinal cord		Medulla oblongata		Motor cortex		Frontal lobe		Cerebellum	
		NCI	GI	NCI	GI	NCI	GI	NCI	GI	NCI	GI
1	C9orf72HRE	++	++	++	++	++	+++	+	+++	++	++
2	C9orf72HRE	++	++	–	+	n.a.	n.a.	–	+	–	+
3	C9orf72HRE	++	++	–	–	n.a.	n.a.	–	–	–	–
4	C9orf72HRE	++	+++	+++	+++	+	+++	+	++	+	++
5	C9orf72HRE	+++	+++	+++	+++	–	+++	++	+++	+	+++
6	C9orf72HRE	++	+++	+	+++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	C9orf72HRE	++	++	++	++	–	++	+	++	+	++
8	C9orf72HRE	++	++	++	++	n.a.	n.a.	+	++	n.a.	n.a.
9	C9orf72HRE	++	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	+
10	C9orf72HRE	++	+++	n.a.	n.a.	+	++	++	++	n.a.	n.a.
11	C9orf72HRE	+++	++	n.a.	n.a.	++	++	++	++	n.a.	n.a.
12	C9orf72HRE	++	++	n.a.	n.a.	–	++	+	++	n.a.	n.a.
13	C9orf72HRE	++	++	++	++	–	++	–	++	–	++
14	C9orf72HRE	+	+	+	++	+	++	–	++	–	++
15	C9orf72HRE	+	++	+	++	+	++	+	++	–	++
16	C9orf72+KIF5A	+++	+++	+++	+++	++	+++	++	+++	+	+++
17	C9orf72+KIF5A	++	++	n.a.	n.a.	n.a.	n.a.	+	+	+	+++
18	C9orf72+KIF5A	++	++	n.a.	n.a.	–	++	++	++	n.a.	n.a.
19	KIF5A	+	–	+	–	–	–	–	–	–	–
20	KIF5A homozyg	++	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
21	KIF5A	+	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	KIF5A	+	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
23	KIF5A	+	++	–	++	–	++	–	++	–	++
24	KIF5A	++	++	n.a.	n.a.	–	+	–	+	–	++
25	NEK1 R812X	++	+++	+++	+++	+	+++	+	+++	n.a.	n.a.
26	VAPB S160delS	+	+++	++	+++	++	+++	++	++	+	+++
27	FUS R495X	++	+++	+	++	–	+	–	+	–	+
28	FUS Q23L	++	++	+	+	n.a.	n.a.	–	++	–	++
29	ALSIN+KIF5A	++	+++	++	+++	++	++	++	++	+	+++
30	SOD1 V5M	++	+	n.a.	n.a.	–	+	n.a.	n.a.	–	–
31	SOD1 A4V+KIF5A	++	++	++	++	–	+	–	+	–	+
32	SOD1 G127X	+	+++	++	+	++	+	–	+	+	+
33	SOD1 G127X	+	+	+	+	n.a.	n.a.	–	+	–	–
34	SOD1 G127X	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
35	SOD1 G114A	+	+	n.a.	n.a.	n.a.	n.a.	–	–	–	–
36	SOD1 G114A	++	++	+	+	+	+	+	++	–	+
37	SOD1 D76Y	++	–	++	–	+	+	+	–	+	–
38	SOD1 D83G	+	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
39	SOD1 D101G	++	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
40	SOD1 L144F	+	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
41	SOD1 D90A	++	+++	++	++	+	+++	–	+	–	+
42	SOD1 D90A	++	+	–	+	–	+	–	+	–	+
43	SOD1 D90A	++	++	++	++	–	+	–	+	–	–
44	SOD1 D90A	++	+++	+	++	–	++	–	++	+	++
45	SOD1 D90A	++	++	++	++	+	++	n.a.	n.a.	–	+
46	SOD1 D90A	++	++	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
47	N.D. Control	–	–	–	–	–	–	–	–	–	–
48	N.D. Control	+	–	–	–	–	–	–	–	–	–
49	N.D. Control	+	–	–	–	–	–	–	–	–	–
50	N.D. Control	+	++	+	++	+	+	–	+	–	++
51	N.D. Control	–	–	–	–	–	–	–	–	–	–
52	N.D. Control	+	–	–	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
53	N.D. Control	–	–	–	–	n.a.	n.a.	–	–	–	–
54	N.D. Control	–	–	–	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Continued

Table 2 Continued

Case	Mutated gene	Spinal cord		Medulla oblongata		Motor cortex		Frontal lobe		Cerebellum	
		NCI	GI	NCI	GI	NCI	GI	NCI	GI	NCI	GI
55	N.D. Control	–	–	–	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
56	N.D. Control	–	–	–	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Rating for misSOD1 inclusions: – no inclusion, + <25% of neurons had inclusions, ++ 25%–75% of neurons had inclusions, +++ >75% of neurons had inclusions. GI, glial inclusion; n.a., not available; NCI, neuronal inclusions; N.D. Control, neurodegenerative control.

and frontal lobe were rarely observed in these patients. In contrast, staining for misSOD1 in patients with ALS heterozygous for the mutations A4V, V5M, D76Y, D83G, D101G, G114A, L144F and G127X - all encoding unstable SOD1 mutants^{11 12 34} revealed larger skein-like and Lewy body-like inclusions in addition to smaller granular misSOD1 inclusions (figures 1J,K–3H,I). Thus, there are (at least) two different morphologies of misSOD1 inclusions present in patients carrying pathogenic mutations in *SOD1*.

The *SOD1*^{G127X} mutation results in a shift in the DNA coding frame and a neopeptide sequence of five amino acids 127GQRWK-stop, truncating after codon 132, 21 amino acids short of end of the SOD1^{WT} polypeptide.¹¹ It is therefore suited for studying different misSOD1 inclusions. Staining with the anti-SOD1 antibody raised against the C-terminal end (131–153 Ra-ab) revealed small and dot-like misSOD1 positive inclusions in the motor neurons that resembled the staining seen in patients with sporadic and familial ALS lacking *SOD1* mutations (figure 2A). This was observed in all three patients heterozygous for this mutation. Double staining with the 131–153 Ra-ab and the mutation-specific antibody G127X Ra-ab revealed *separate* inclusions of misSOD1 in motor neurons, without colocalisation (figure 2A–C): the large skein-like inclusions contain the misfolded SOD1^{G127X} mutant protein, the small granular dot inclusions the misSOD1^{WT} generated from the wild-type allele. Both types of inclusions in the same motor neuron were only observed in a few motor neurons per section. Most motor neurons had only one type of SOD1 inclusion and a few neurons lacked inclusions.

Control patients and reanalysis of spinal cord sections from patients with ALS and controls

To determine whether the misSOD1^{WT} inclusions were specific for patients with ALS and FTD, brain and spinal cord tissue from 10 control patients with other neurodegenerative diseases were stained with the same anti-misSOD1 antibodies. Nine of 10 controls did not show misSOD1^{WT} inclusions anywhere in the spinal cord or brain (table 2, figures 1I and 3F). A single patient diagnosed with epilepsy after a cerebral infarction had some misSOD1^{WT} inclusions in the spinal cord and hypoglossal nucleus. Neuronal cytoplasmic misSOD1^{WT} inclusions were also seen to the same extent as in the motor cortex of patients with ALS, but the frontal cortex and cerebellum were blank. We additionally analysed spinal cord sections from cervical, thoracic and lumbar regions of 20 patients with non-neurological diseases (online supplementary table 1) with the anti-misSOD1 antibodies. No inclusions of misSOD1^{WT} were observed in any of these (figure 2E).

The above reported IHC investigations were carried out over a period of 14 years. During this time, the staining equipment was changed in our lab. For control purposes, we recently restained and re-examined in parallel lumbar spinal cord specimen from all 30 controls and 38 (of the 46) patients with ALS at the same time (CNS tissues were no longer available from two patients with *C9orf72* and six patients with *SOD1* mutations). Two different antibodies, 57–72 Ra-ab and 131–153 Ra-ab—targeting the active site and the carboxy end of the SOD1 protein, respectively—were used to ascertain the specificity for misSOD1 in the stainings. Identical protocol was used for all specimen.

Table 3 MisSOD1 immunohistochemical results

A	Number of patients with misSOD1 inclusions in spinal cord motor neurons				
	SOD1 fALS	C9orf72HRE expansion	Other ALS mutations	Controls, neurodegenerative	Controls, non-neurological
Cervical spinal cord	17 (17)	17 (18)	11 (11)	1 (10)	0 (14)
Thoracic spinal cord	16 (16)	15 (15)	11 (11)	1 (3)	0 (18)
Lumbar spinal cord	16 (16)	15 (16)	11 (11)	4 (10)	2 (19)
B	Proportion of motor neurons with misSOD1 staining				
	SOD1 fALS	C9orf72HRE expansions	Other ALS mutations	Controls, neurodegenerative	Controls, non-neurological
Cervical spinal cord	+ (+ – ++)	++ (0 – +++)	++ (+ – ++)	0 (0 – ++)	0 (0)
Thoracic spinal cord	+ (+ – ++)	++ (+ – +++)	++ (+ – ++)	0, 0, ++*	0 (0)
Lumbar spinal cord	+ (+ – ++)	++ (0 – +++)	++ (+ – ++)	0 (0 – +)	0 (0 – +)

Table of findings from sections stained with the 131–153 Ra-ab and 57–72 Ra-ab. Data for number of patients with misSOD1^{WT} inclusions show the total number of patients in parenthesis. Data for proportion of misSOD1^{WT} inclusions with staining are shown as median (range) referring to a four-tiered semiquantitative scale (0=no glial or motor neuron with staining; + ≤25% of the motor neurons with staining; ++=25%–75% of the motor neurons with staining; +++ ≥75% of the motor neurons with staining). The total number of patients in each group was as follows: 17 *SOD1*, 18 *C9orf72HRE*, 11 other ALS mutations (2 with *FUS*, 1 *ALSIN+KIF5A*, 1 *VAPB*, 1 *NEK1*, 6 *KIF5A*); 10 Controls neurodegenerative, 20 Controls non-neurological. Sections from all levels were not available from all patients. In total, of the 20 non-neurological controls, only two had some staining. *Since thoracic spinal cord only was available from three patients from the neurodegenerative control group each result is presented individually. ALS, amyotrophic lateral sclerosis; fALS, familial ALS.

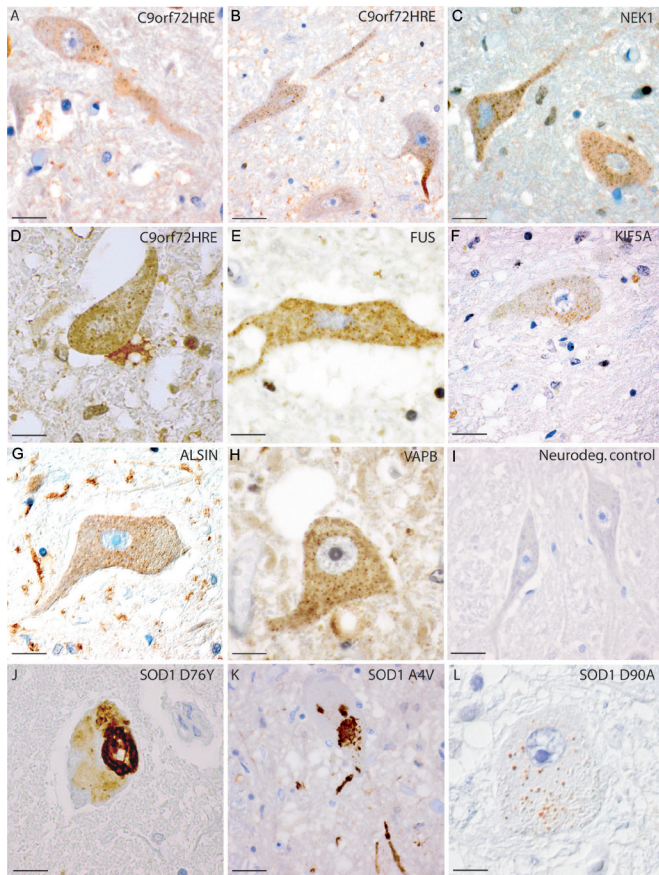


Figure 1 Inclusions of misSOD1^{WT} in spinal cord sections of patients with mutations in six ALS/FTD-associated genes. MisSOD1^{WT} was present in histological sections from patients with ALS carrying *C9orf72HRE* (A–B, cases #2 and #8), *NEK1* mutation (C, case #25), *C9orf72HRE* mutation (D, case #16), *FUS* mutation (E, case #27), *KIF5A* mutation (F, case #19), *alsin* mutation (G, case #29), *VAPB* mutation (H, case #26), *SOD1*^{D76Y} mutation (J, case #37), *SOD1*^{A4V} mutation (K, case #31) and *SOD1*^{D90A} homozygous mutation (L, case #43), but not in a neurodegenerative control case (I, case #47). All sections were stained with the 131–153 Ra-ab peptide antibody. In panels (A–H), multiple small granular cytoplasmic inclusions of misSOD1^{WT} can be seen in motor neurons with a size of approximately 0.5–3 μm. In panel L, staining of the wt-like *SOD1*^{D90A} mutant is shown. Note the morphological similarity between panel L and panels A–H, where misSOD1^{WT} is stained. In panels J and K, the *SOD1* mutations D76Y and A4V, which give rise to unstable SOD1 proteins, are found to show skein-like cytoplasmic SOD1 inclusions in motor neurons. Scale bars: A–F and I 50 μm; G–H, J–L 30 μm. ALS, amyotrophic lateral sclerosis.

The identities of the restained sections were coded and were independently examined by two neuropathologists (KF, TB).

The results are summarised in figure 4, online supplementary figure 2 and supplementary table 2. The two examiners assessed the sections in principle equally, the only differences being a few one step differences in gradings of the SOD1 stainings in patients with ALS, online supplementary table 2. In these cases, both gradings are shown. All the patients with ALS without *SOD1* mutations again showed the misSOD1^{WT}-positive granular inclusions. They were easily detected and had the same shape and locations as seen in the previous older stainings. The misSOD1^{WT} staining in the neurodegenerative control #50 was again seen (online supplementary figure 2 AI). However, the principal difference from the previous analyses was that misSOD1^{WT} staining was now seen in three more neurodegenerative control (online

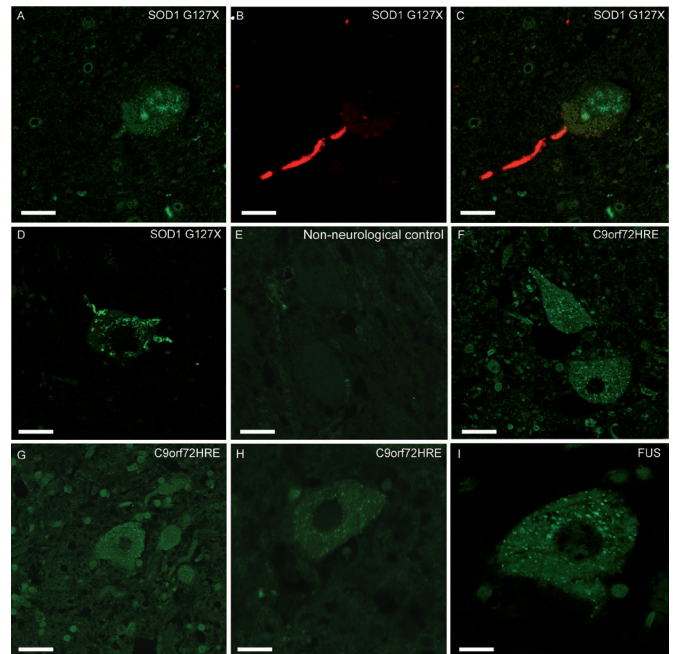


Figure 2 Inclusions of misSOD1 observed in human spinal cord motor neurons. Micrographs showing lumbar ventral horn motor neurons from two patients with the *SOD1*^{G127X} mutation (A–C, case #33 and D, case #32), a non-neurological control (E, case #64), three patients with *C9orf72HRE* mutation (F–H, cases #1, #11 and #5) and a *FUS* mutation (I, case #27). Panels A–C show sections double-labelled with the 131–153 Ra-ab peptide antibody which stains a segment of the SOD1 protein that is absent in the *SOD1*^{G127X} mutant, and therefore only stains misSOD1^{WT} (A, C, green fluorescence) and the G127X Ra-ab which only recognises the mutated G127X sequence (B, C, red fluorescence). The merged picture of green and red channel scans show that this neuron carries inclusions of both mutated SOD1 (seen here in the axon as red) and of misSOD1^{WT} (seen here in the soma as green). The mutated and misSOD1^{WT} inclusions do not colocalise (C). Panel D was stained with the G127X Ra-ab and panels E–I were stained with the 57–72 Ra-ab peptide antibody. Note the small dot-like granular cytoplasmic inclusions of misSOD1 in motor neurons (F–I) which is not seen in the non-neurological control (E). The mutated, unstable *SOD1*^{G127X} protein has more of a skein-like appearance (D). Scale bars: A–G, 50 μm, H 30 μm, I 20 μm.

supplementary figure 2 AG, AH, AK), which did not appear in the previous stainings (table 2). Two of these patients had Parkinson's disease and one had Alzheimer's disease. Noticeably, only a few motor neurons were positive. Moreover, also two of the non-neurological controls were when restained now graded as positive since a few motor neurons were positive (online supplementary figure 2 AQ, AW).

The outcomes of examinations of sections stained with two different anti-SOD1 peptide antibodies (57–72 Ra-ab, 131–153 Ra-ab) were almost identical (figure 4 and online supplementary table 2). This proves that the component that becomes stained in the granules is indeed misSOD1^{WT}. The 131–153 Ra-ab gives generally less background staining and was therefore chosen for most of the histochemical panels shown.

DISCUSSION

We here report the finding of misSOD1^{WT}-positive intracytoplasmic inclusions in motor neurons of patients lacking *SOD1* mutations but carrying pathogenic mutations in six other ALS-causing genes *C9orf72HRE*, *FUS*, *NEK1*, *KIF5A*, *ALSIN*

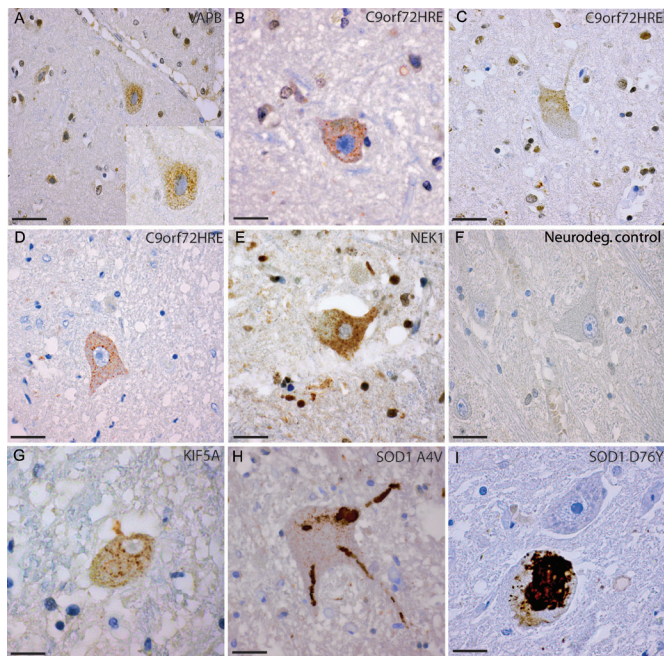


Figure 3 Histological sections from the motor cortex and medulla oblongata of patients carrying different ALS-causing mutations. Sections were stained with the 131–153 Ra-ab peptide antibody. Panels A–C show Betz cells in the motor cortex of patients with a *VAPB* mutation (A, case #26) and *C9orf72HRE* mutation (B, case #13 and C, case #16). Small dot-like cytoplasmic inclusions of misSOD1^{WT} are apparent in the Betz cells (A–C). Panels D–I show neurons in the medulla oblongata of patients with *C9orf72HRE* (D, case #15), *NEK1* mutation (E, case #25), a neurodegenerative control (F, case #55), *KIF5A* mutation (G, case #19), the *SOD1*^{A4V} mutation (H, case #31) and the *SOD1*^{D76Y} mutation (I, case #37). In panels D, E and G, inclusions of granular misSOD1 are observed in neurons of the hypoglossal nucleus. Note the mutated SOD1 protein, which appears skein-like in panels H–I compared with the granular misSOD1^{WT} protein (appearing as granular intracytoplasmic dots in panel D (E and G)). Scale bars: A 100 µm; B–G 50 µm, H–I 30 µm. ALS, amyotrophic lateral sclerosis.

and *VAPB*. The inclusions were small, numerous and scattered in the cytoplasm of motor neurons at all levels of the spinal cord, in the motor nuclei of the brain stem and occasionally in the motor and frontal cortex. They were seen in all 18 patients carrying *C9orf72HRE*, regardless of whether the patient had a ‘pure’ FTD diagnosis, pure ALS diagnosis or both. The morphology and shape of these inclusions resembled that of the inclusions described in sALS.¹⁷ Patients with other FTD-associated and ALS-associated mutations had similar inclusions that stained for misSOD1^{WT} in the brain and spinal cord. The finding that misSOD1^{WT} appears in small inclusions in all studied patients with ALS raises the question if these misSOD1^{WT} inclusions are different from the ones formed from mutated SOD1.

In *SOD1* Tg murine models overexpressing the G85R, G93A, D90A or G127X human *SOD1* mutations, larger skein-like inclusions are observed when stained with human misSOD1-specific antibodies. The misSOD1 in these *SOD1* Tg murine models is aggregated. So far, two strains of aggregates (A and B) have been described which differ with regard to molecular structure.¹⁴ Both display prion-like properties and propagate further aggregation and phenotypically different ALS-like diseases when inoculated into the spinal cord of 100-day-old asymptomatic mice Tg for the human *SOD1*^{G85R} mutation.¹⁵ In patients that carry unstable

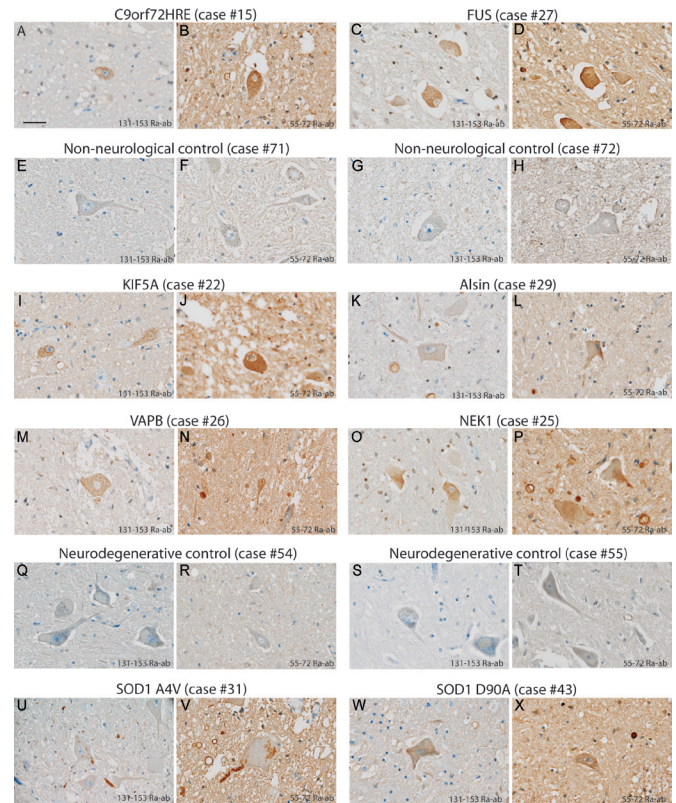


Figure 4 Restaining of misSOD1^{WT} inclusions in lumbar spinal cord sections from patients carrying different ALS/FTD associated gene mutations as well as neurodegenerative and non-neurological controls. Micrographs show (A,B) *C9orf72HRE* (case #15), (B,C) *FUS* (case #27), (E,F) non-neurological control (case #71), (G,H) non-neurological control (case #72), (I,J) *KIF5A* (case #22), (K,L) *alsin* (case #29), (M,N) *VAPB* (case #26), (O,P) *NEK1* (case #25), (Q,R) neurodegenerative control (case #54), (S,T) neurodegenerative control (case #55), U–V *SOD1*^{A4V} (case #31), W–X *SOD1*^{D90A} (Case #43). For comparison, sections were stained with either the 131–153 Ra-ab (A, C, E, G, I, K, M, O, Q, S, U and W) or the 57–72 Ra-ab (B, D, F, H, J, L, N, P, R, T, V and X). Multiple small dot-like inclusions are seen with both antibodies in all patients carrying different ALS/FTD associated gene mutations but are absent in the neurodegenerative and non-neurological controls. Note the high background staining when using 57–72 Ra-ab. Scale bars: A–X 100 µm for all micrographs.

mutant SOD1s, morphologically similar misSOD1-IR inclusions are found.¹¹ These also appear to contain aggregated SOD1, but the molecular structures have yet to be elucidated. Inoculation of *SOD1*^{G127X} aggregates purified from the spinal cord of patient no. 32 in this study (table 1) into the lumbar spinal cord of 100-day-old mice induced hSOD1-aggregate formation throughout the neuraxis concomitant with onset of ALS.¹⁶ Binary epitope mapping revealed that the human misSOD1 aggregates was of the A-type.¹⁶ Further inoculation studies of *SOD1* aggregates purified from other patients from this study are ongoing.

In patients homozygous for the stable wt-like mutant *SOD1*^{D90A}, the motor neurons display small granular misSOD1^{D90A} inclusions. Whether these also have prion-like features as the larger inclusions found in the murine Tg models and in patients that carry unstable mutant SOD1s, remains to be determined. Still, this is the sole conspicuous misSOD1-IR structure found in post-mortem tissue from the six *SOD1*^{D90A} homozygous patients. The differences in morphology compared with patients with other *SOD1* mutations might be related to their different molecular

stabilities. SOD1^{WT} and stable SOD1 mutants might associate to form small granular inclusions whereas unstable mutant proteins could either be rapidly degraded or form fewer but larger inclusions. Interesting implications follow from this observation: (1) inclusions of misSOD1 may be pathological hallmarks in all forms of ALS and (2) variation in morphology, distribution and number of aggregates may be used to distinguish different forms and stages of ALS.

Summarising the results of the initial staining and the restaining, 4 of 10 neurodegenerative controls had some inclusions of misSOD1^{WT} (online supplementary figure 2 AG-AI, AK, and supplementary table 2). One of the positive controls was a patient with epilepsy after cerebral infarction. Here, small granular cytoplasmic inclusions of misSOD1^{WT} were seen in spinal cord motor neurons (online supplementary figure 2 AI), in the hypoglossus nuclei and in the motor cortex. No inclusions were found in the striatum, frontal cortex or mesencephalon. Speculatively, this control individual could have had clinically unrecognised early ALS and/or the cerebral infarction triggered a cascade of events resulting in misfolding of misSOD1^{WT} that might not have prion-competent conformations¹⁵ but are still detected by our polyclonal antibodies. However, cerebrovascular injury has been proposed to be a risk factor for ALS.³⁵ Two of the positive controls had Parkinson's disease (online supplementary figure 2 AG, AK). Interestingly, inclusions of misSOD1^{WT} have been found to accumulate in the brains of patients with Parkinson's disease and suggests common mechanisms of misSOD1^{WT} aggregation in both disorders.³⁶ Moreover, neurodegenerative diseases with concomitant copathology is frequent.³⁷

Two of the non-neurological controls also had a few motor neurons that stained positive for SOD1. One died of a dissection of the aorta and the other of myocardial infarction (online supplementary figure 2 AQ, AW). The first showed small areas of infarction in the spinal cord. As also noted earlier in senile plaques, the state of the tissue might thus affect the immunoreaction found. All other 24 controls stained negative for misSOD1 in all studied tissue specimens, demonstrating major differences between patients with ALS and controls.

The presence of misSOD1^{WT} in postmortem CNS tissue of sALS and fALS without SOD1 mutations is controversial: some studies support the result of the present study,^{17–22} but in other studies misSOD1 could not be detected in sALS^{38–40} or there were no differences between sALS and control individuals.^{41–42} Reasons for these opposite results were discussed in a recent article²² and includes methodological differences (ie, the use of TRIS/EDTA buffers instead of citrate-based buffers, different preanalytical tissue handling, variable incubation times, different antibody dilutions). Guidelines for staining misSOD1 were proposed.²² These guidelines were adhered to in this work. It should be noted that Ref. 21 was a blinded study of sALS cases compared with controls (some of these were the same as in this study) and using some of the anti-misSOD1 antibodies as in the present study.

The present morphological study does not prove that misSOD1^{WT} causes neurodegeneration in patients carrying mutations in *C9orf72HRE*, *FUS*, *NEK1*, *KIF5A*, *ALSIN* or *VAPB*. Nor can we explain how patients with a massive intronic GGGGCC repeat-expansion in *C9orf72* or a R495X-truncation in the mRNA-housekeeping *FUS* enzyme have inclusions of misSOD1^{WT} in their motor neurons and with a morphology and similar pattern of cellular distribution as in patients with the SOD1^{D90A} mutation. We speculate that whatever the initial pathogenic events are in patients carrying *C9orf72HRE* or coding mutations in *FUS*, *NEK1*, *KIF5A*, *VAPB* or *ALSIN*, eventually they

converge to common downstream event(s) involving unfolding and misfolding of SOD1^{WT}. For this to happen, the stabilising intrasubunit C57-C146 disulfide bond must be reduced and the stabilising Zn²⁺ cofactor released. If the generated misfolded SOD1 species are pathogenic and form SOD1^{WT} prions, based on injection of SOD1 aggregates,^{15–16} this event may only have to occur in a limited number of cells, perhaps only once for the SOD1-prion cascade to be initiated. The finding that over-expression of human SOD1^{WT} in mice elicits a fatal ALS-like disease supports that SOD1^{WT} can cause neurodegeneration.²³ The observation that all three patients heterozygous for the SOD1^{G127X} mutation had inclusions with the truncated mutant SOD1^{G127X} protein (easily recognisable because of its neopeptide sequence) and separate inclusions containing only misSOD1^{WT} (detectable by the amino acid sequence that is missing in the mutated SOD1 protein) is interesting. It raises the possibilities that either the misfolding of SOD1^{WT} is a secondary non-pathogenic event in a stressed cell or that misfolded SOD1^{WT} participates and could be pathogenic per se, raising the possibility that there could be more than one kind of SOD1 prions active simultaneously in patients with SOD1 mutations. Simply targeting a mutant SOD1 gene with tailored genetic inhibition therapy may therefore not be sufficient to halt disease progression. This observation is important, since new therapies targeting SOD1 are ongoing or are being planned. If SOD1 plays a role in subtypes of ALS without SOD1 mutations as our results suggest, then many patients may benefit from these new anti-SOD1 therapeutics.

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Contributors Study concept: PA. Designed the study: KF, SM, TB, PA. Collected and evaluated the material: KF, KG, BP, MW, MN, TB, PA. Performed and evaluated the laboratory analysis: KF, MW, MN, SLM, TB. Wrote the manuscript: KF, TB, PA.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved by the Regional Medical Ethical Review Board in Umea, Sweden with applications from 1994 (FEK no. 94-135) and 2014 (EPN no. 14-17-31M).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement There are additional pictures of misSOD1+ stainings available from the here studied material which we are willing to share.

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