Full title: Altered SOD1 maturation and post-translational modification in amyotrophic lateral sclerosis spinal cord

Short title: SOD1 biochemistry in post-mortem ALS

SUPPLEMENTARY INFORMATION

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Supplementary Table 1. Demographic and clinical information for human post-mortem tissue cases.

Case #	Diagnostic Group	Age	Sex	PMI	Site of onset	Cause of Death	Fixed Tissue Regions	Fresh Frozen Tissue Regions
1	Age-matched Control	51	F	33	N/A	Lung Cancer	TSpC	TSpC, OCx
2	Age-matched Control	54	M	30	N/A	Cancer	TSpC	TSpC, OCx
3	Age-matched Control	73	F	27	N/A	NA	TSpC	TSpC, OCx
4	Age-matched Control	65	M	25	N/A	Ruptured Aortic Aneurysm	TSpC, CSpC	TSpC, CSpC, OCx
5	Age-matched Control	50	M	24	N/A	Atherosclerotic Cardiovascular Disease	TSpC	TSpC, CSpC, OCx
6	Age-matched Control	48	M	22	N/A	Hypertensive Atherosclerosis Heart Disease	TSpC, CSpC	TSpC, CSpC, OCx
7	Age-matched Control	49	F	26	N/A	Hypertensive Atherosclerosis Heart Disease	TSpC	TSpC, CSpC, OCx
8	Age-matched Control	55	M	23	N/A	Cardiac Arrythmia (Cardiomegaly)	TSpC, CSpC	TSpC, CSpC, OCx
9	Age-matched Control	49	M	22	N/A	Cardiac Tamponade	TSpC, CSpC	TSpC, CSpC, OCx
10	Age-matched Control	50	M	22	N/A	Hypertensive Atherosclerosis Heart Disease	TSpC, CSpC	TSpC, CSpC, OCx
11	fALS (SOD1, I113T)	47	M	13.5	Bulbar	Complication of Disorder	TSpC	TSpC, OCx, OCx
12	fALS (SOD1, I113T)	70	F	56	Bulbar	Complication of Disorder	TSpC	TSpC, OCx, OCx
13	fALS (SOD1, D101G)	46	F	5	Limb - upper	Complication of Disorder	TSpC	TSpC, OCx, OCx
14	fALS (C9ORF72, 30+ positive)	64	M	68	Bulbar	Complication of Disorder	TSpC	TSpC, OCx
15	fALS (C9ORF72, 30+ positive)	62	M	20	Bulbar	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
16	fALS (C9ORF72, 30+ positive)	55	M	24	Limb - upper (right)	Complication of disorder	TSpC, CSpC	TSpC, CSpC, OCx
17	fALS (unknown mutation)	65	M	7	Limb - lower (left)	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
18	sALS	70	F	15	Limb - upper	Complication of Disorder	TSpC	TSpC, OCx
19	sALS	50	F	56	Limb - lower	Complication of Disorder	TSpC	TSpC, OCx

20	sALS	68	F	17	Limb - lower	Complication of Disorder	TSpC	TSpC, CSpC, OCx
21	sALS	54	F	12	Limb - upper and lower	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
22	sALS	56	M	22	Limb - lower	Complication of Disorder	NA	TSpC, CSpC, OCx
23	sALS	68	M	4	Limb - upper and lower	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
24	sALS	57	F	20	Limb - lower (right)	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
25	sALS	62	M	4	Bulbar	Complication of Disorder	TSpC, CSpC	TSpC, CSpC, OCx
26	sALS	70	F	29	Limb - lower	Complication of Disorder	NA	TSpC, CSpC, OCx

Abbreviations: CSpC, cervical spinal cord; fALS, familial amyotrophic lateral sclerosis; F, female; M, male; MRC, MRC London Neurodegenerative Diseases Brain Bank; NIH, National Institute of Health NeuroBioBank; N/A, not applicable; NA, not available; OCx, occipital cortex; PMI, post-mortem interval; sALS, sporadic amyotrophic lateral sclerosis; TSpC, thoracic spinal cord.

Supplementary Table 2. Demographic statistics for diagnostic groups.

	Diagnostic group				Significantly different?
	Control	**SOD1-fALS**	**Non-SOD1-fALS**	**sALS**	
n	10	3	4	9	N/A
Sex (M:F)	7:3	1:2	4:0	4:5	CD[a]
Age (years)	54.4±2.6	54.3±7.8	61.5±2.3	61.67±2.6	N.S. (p = 0.14)[b]
[range]	[48-73]	[46-70]	[55-65]	[50-70]	
PMI (hrs)	25.4±1.2	24.8±15.8	29.8±13.3	19.9±5.3	N.S. (p = 0.23)[b]

Footnotes: [a] Chi-square test, [b] Kruskal-Wallis test

Abbreviations: CD, could not determine; fALS, familial amyotrophic lateral sclerosis; F, female; M, male; N/A, not applicable; N.S., not significant; PMI, post-mortem interval; sALS, sporadic amyotrophic lateral sclerosis.

Supplementary Table 3. Primary antibody details and applications.

Antibody	Source (cat#)	Class	Host	Species reactivity	Immunogen	Application	Dilution
$SOD1_1$	Merck Millipore, Billerica, MA, USA (574597)	P	Sh	Hu, Mn, Rat, Ms	Highly purified, human erythrocyte SOD1	IHC	1:200
$SOD1_2$	Biolegend, San Diego, CA, USA (850701)	M	Ms	Hu, Ms, Rat	Purified full length human SOD1 expressed in 293T cells	IB IHC	1:5,000 1:500
$SOD1_3$	Enzo Life Sciences, Farmingdale, NY, USA (ADI-SOD-100)	P	Rb	Hu, Ms, Rat	Native human Cu/Zn SOD	IP	N/A
DisSOD1 (B8H10)	Medimabs Inc, Quebec, Canada (MM-0070-P)	M	Ms	Hu	Recognizes misfolded forms of mutant human SOD1 protein	IHC	1:200
DisSOD1 (EDI)	StressMarq Biosciences, British Columbia, Canada (SPC-206)	P	Rb	Hu, Ms, Rat	N-terminal region, SOD1 protein with exposed dimer interface	IHC	1:200
DisSOD1 (UβB)	StressMarq Biosciences, British Columbia, Canada (SPC-205)	P	Rb	Hu, Ms, Rat	N-terminal region, SOD1 protein with unfolded β-barrel	IHC	1:200
CCS_1	Santa Cruz, Dallas, Texas, USA (sc-55561)	M	Ms	Hu, Ms, Rat	Amino acids 1-274 representing full length CCS of human origin.	IHC IB	1:100 1:1,000
CCS_2	Sigma-Aldrich, St. Louis, MO, USA (SAB1406905)	P	Ms	Hu	Amino acids 1-274 representing full length CCS of human origin.	IHC	1:100
GFAP	Sigma-Aldrich, St. Louis, MO, USA (G3893)	M	MS	Hu, Rat	GFAP from pig spinal cord	IB	1:30,000
TUJ-1	Biolegend, San Diego, CA, USA (801213)	M	Ms	Hu, Ms, Rat	The last 15 C terminal residues of neuron-specific β3-tubulin	IHC	1:500

Abbreviations: CCS, copper chaperone for SOD1; DisSOD1, structurally-disordered SOD1; Hu, human; IB, immunoblotting; IHC, immunohistochemistry; IP, immunoprecipitation; Mn, monkey; M, monoclonal; Ms, mouse; P, polyclonal; Rb, rabbit; Sh, sheep; SOD1, superoxide dismutase 1.

Supplementary Table 4. **Voltage and current settings for native isoelectric focussing experiments.**

Step	**Voltage (V)**	**Rapid/slow ramp**	**Time / kVhrs**
1	200	Rapid	20 min
2	450	Rapid	15 min
3	750	Rapid	15 min
4	2000	Rapid	12 kVh
5	200	Rapid	12 hrs

Supplementary Table 5. Chromatographic gradient employed for the separation of peptides using high performance liquid chromatography prior to tandem mass spectrometry. Solvent A, 0.1% formic acid in MilliQ water; solvent B, 0.1% formic acid in 80% ACN diluted with MilliQ water.

Step	Time (min)	Flow (nL/min)	%B
1	0	450	5
2	11	450	5
3	11.1	300	5
4	13	300	5
5	70	300	50
6	75	300	98
7	78	450	98
8	89	450	98
9	90	450	5

Supplementary Table 6. Parameters for the quantification of SOD1 peptides by parallel reaction monitoring (PRM) and liquid chromatography-mass spectrometry (LC-MS). Free thiol (NEM) and reversibly modified (MMTS) Cys peptides were quantified by PRM. Unmodified peptides were monitored for normalisation; Cys Modification, alkylating agent present at Cys residue; Precursor mass, mass of the modified peptide precursor ion in daltons (Da); Precursor charge, charge state of modified peptide precursor ion; Precursor m/z, mass to charge ratio of modified peptide precursor ion; Transitions quantified, fragment ions originating from modified precursor ion used for quantitation, charge state of transition denoted by +.

Peptide Sequence	Cys Modification	Precursor Mass (Da)	Precursor Charge	Precursor m/z	Transitions Quantified
GLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSR	MMTS	3511.613	4	877.903	y15+, y14+, y12+, y11+, y10+, y9+, y7+, y6+, y5+, y4+, y2+, y26++, y23++, y22++, y21++, y20++, y19++, y18++, y17++, y16++, y15++, y14++, y13++, y12++, y11++, y10++, y9++, y8++, y7++, b3+, b5+, b6+, b7+, b8+, b9+, b10+, b11+, b12+, b13+, b14+, b8++, b11++, b12++, b13++, b14++, b15++, b16++, b17++, b18++, b19++, b20++, b21++, b25++
GLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSR	NEM	3590.673	4	897.668	y9+, y5+, y22++, y21++, y19++, y17++, y15++, y14++, y9++, y8++, b7+, b10+, b11+, b12++, b13++, b21++
LACGVIGIAQ	MMTS	991.518	2	495.759	y4+, y2+, y1+, b4+, b5+, b7+
LACGVIGIAQ	NEM	1070.578	2	535.289	y2+, y1+, b4+, b5+, b7+
GDGPVQGIINFEQK	None	1502.772	2	751.386	y10+, y9+, y8+, y7+, y6+, y5+, y3+, y2+, y11++, b2+, b3+, b4+, b5+, b6+, b8+, b3++, b8++
HVGDLGNVTADK	None	1226.624	2	613.312	y11+, y10+, y8+, y4+, b1+, b2+, b7+

Supplementary Table 7. Immunoprecipitation did not alter levels of post-translational modifications of interest on a commercially available human SOD1 standard. Statistical comparisons were performed using pairwise Mann-Whitney U tests for each PTM of interest at each residue, between protein aliquots that had (n = 3), and had not (n = 4), been immunoprecipitated.

PTM	Residue	Mann-Whitney U	p value
3DG-H (Arg)	Arg115	0	0.13
	Arg69	3	>0.99
	Arg143	2	0.23
Acetylation (Lys)	Lys3	4	>0.99
	Lys9	3	0.40
	Lys30	0	0.06
	Lys91	2	0.40
	Lys122	2	0.40
CEL (Lys)	Lys136	0	0.13
	Lys9	3	0.40
	Lys70	2	0.23
CML (Lys)	Lys9	1	0.20
	Lys91	2	0.23
	Lys122	0	0.06
Deamidation (Asn, Gln)	Asn131	3	0.40
	Asn139	1	0.11
	Asn19	0	0.06
	Asn26	3	0.40
	Asn86	3	0.40
	Asn53	0	0.06
	Asn65	2	0.53
	Gln15	2	0.23
	Gln22	0	0.06
MG-H (Arg)	Arg143	3	0.40
	Arg69	0	0.06
	Arg115	2	0.23
Nitration (Trp)	Trp32	5	0.86
Oxidation (His, Trp)	His80	0	0.06
	His110	5	0.86
	His43	4	0.63

	His46	5	0.86
	His48	4	0.63
	His63	1	0.11
	His120	3	0.40
	Trp32	1	0.11
Succinylation (Lys)	Lys3	2	0.53
	Lys9	5	0.86
	Lys91	2	0.40
	Lys122	1	0.40
Ubiquitination (Lys)	Lys91	4	>0.99

Abbreviations: 3 DG-H, 3-deoxyglucosone-derived hydroimidazolone; CEL, carboxyethyllysine; CML, carboxymethyllysine; MG-H, methylglyoxal-derived hydroimidazolone.

Supplementary Table 8. Complete details of statistical tests used to analyse PTMs of interest between ALS subgroups and controls.

PTM	Residue	Tissue region	Significant variation between groups?	Significant differences in pairwise comparisons?	Test employed	Test statistic(s)	p value(s) - ANOVA (post-hoc)	Fold change vs control
3 DG-H	Arg115	VSpC	No	No	ANOVA	F = 1.764	0.19	N/A
AAAA	Lys70	VSpC	No	No	Kruskal-Wallis	H = 3.763	0.29	N/A
Acetylation	Lys3	VSpC	Yes	Yes - ↓ sALS vs Ct	Kruskal-Wallis w Dunn's	H = 7.494	**0.01 (0.03)**	1.9
		DSpC		**PTMs not identified**				
	Lys9	VSpC	No	No	Kruskal-Wallis	H = 0.739	0.71	N/A
	Lys91	VSpC	No	No	Kruskal-Wallis	H = 1.500	0.77	N/A
Allysine	Lys70	VSpC	No	No	ANOVA	F = 1.199	0.34	N/A
	Lys91	VSpC	No	No	ANOVA	F = 0.4433	0.72	N/A
CEL	Lys9	VSpC	No	No	Unpaired t-test	t=0.8771, df=5	0.42	N/A
	Lys122	VSpC	No	Yes - ↑ SOD1-fALS vs Ct	ANOVA, Mann-Whitney	F = 2.034 (U = 0)	0.15 **(0.036)**	2.04
		DSpC		**PTMs not identified**				
	Lys128	VSpC	No	Yes - ↑ SOD1-fALS vs Ct	ANOVA, Mann-Whitney	F = 2.034 (U = 0)	0.15 (**0.036**)	2.04
		DSpC		**PTMs not identified**				

CML	Lys9	VSpC	No	No	ANOVA	F = 0.4420	0.73	N/A
	Lys70	VSpC	No	No	Kruskal-Wallis	H = 4.642	0.21	N/A
	Lys91	VSpC	No	No	Kruskal-Wallis	H = 2.170	0.54	N/A
	Lys122	VSpC	No	No	Kruskal-Wallis	H = 2.280	0.55	N/A
	Lys128	VSpC	No	No	Kruskal-Wallis	H = 1.286	0.91	N/A
Cysteine redox	Cys57 (MMTS[a])	VSpC	Yes	Yes - ↑ cluster vs Ct	ANOVA w Dunnet's	F = 8.075	**<0.0001 (<0.0001)**	2
	Cys57 (NEM[b])	VSpC	Yes	Yes - ↓ cluster vs Ct	Kruskal-Wallis w Dunn's	H = 9.373	**0.03 (0.032)**	8.7
	Cys146 (MMTS[a])	VSpC	Yes	Yes - ↑ cluster vs Ct	ANOVA w Dunnet's	F = 63.2	**<0.0001 (<0.0001)**	4
	Cys146 (NEM[b])	VSpC	No	Yes - ↓ cluster vs Ct	Kruskal-Wallis w Dunn's	H = 8.792	**0.08 (0.04)**	7.2
Deamidation	Gln15	VSpC	Yes	Yes - ↑ SOD1-fALS vs Ct	ANOVA w Dunnet's	F = 3.366	**0.04 (0.039)**	2.23
		DSpC	No	No	ANOVA w Dunnet's	F = 1.174	0.34 (>0.99)	N/A
	Asn19	VSpC	Yes	No	Kruskal-Wallis w Dunn's	H = 8.314	**0.04** (0.075)	N/A
	Gln22	VSpC	Yes	No	ANOVA w Dunnet's	F = 4.732	**0.012**	N/A

	Asn26	VSpC	Yes	Yes - ↑ SOD1-fALS vs Ct	ANOVA w Dunnet's	F = 5.180	**0.0074 (0.037)**	1.94
		DSpC	No	No	Kruskal-Wallis w Dunn's	H = 4.359	0.23 (0.21)	N/A
	Asn53	VSpC	Yes	Yes - ↑ SOD1-fALS vs Ct	ANOVA w Dunnet's	F = 31.20	**<0.0001 (<0.0001)**	10.18
		DSpC	No	No	Kruskal-Wallis w Dunn's	H = 4.868	0.18 (0.12)	N/A
	Asn65	VSpC	No	No	Kruskal-Wallis w Dunn's	H = 5.222	0.12	N/A
	Asn86	VSpC	No	No	Kruskal-Wallis	H = 4.497	0.21	N/A
	Asn131	VSpC	Yes	Yes - ↑ SOD1-fALS vs Ct	ANOVA w Dunnet's	F = 7.802	**0.0011 (0.0005)**	12.71
		DSpC	No	No	Kruskal-Wallis w Dunn's	H = 3.670	0.30 (0.57)	N/A
	Asn139	VSpC	Yes	No	Kruskal-Wallis w Dunn's	H = 7.267	**0.042**	N/A
Nitration	Trp32	VSpC	Yes	Yes - ↑ sALS vs Ct	Kruskal-Wallis w Dunn's	H = 8.209	**0.0043 (0.019)**	3.41
		DSpC	No	No	Kruskal-Wallis w Dunn's	H = 0.904	0.85 (>0.99)	N/A
Oxidation	His43	VSpC	No	No	ANOVA	F = 0.6673	0.52	N/A

	His46	VSpC	No	No	ANOVA	F = 2.783	0.066	N/A
	His48	VSpC	Yes	Yes - ↑ Zn-def. hSOD1 vs Ct	ANOVA w Dunnet's	F = 7.563	**0.0032 (0.044)**	1.8
		DSpC	No	No	ANOVA w Dunnet's	F = 1.862	0.17 (0.11)	N/A
	His63	VSpC	Yes	Yes - ↑ Zn-def. hSOD1 vs Ct	ANOVA w Dunnet's	F = 10.44	**0.0006 (0.013)**	1.9
		DSpC	Yes	Yes - ↑ SOD1-fALS vs Ct	ANOVA w Dunnet's	F = 3.322	**0.039 (0.015)**	2.3
	His80	VSpC	No	No	ANOVA	F = 1.385	0.27	N/A
	His110	VSpC	No	No	ANOVA	F = 2.058	0.15	N/A
	His120	VSpC	No	No	ANOVA	F = 0.4128	0.67	N/A
	Trp32	VSpC	No	Yes - ↑ SOD1-fALS vs Ct	Unpaired t-test	t=2.265, df=10	**0.047**	1.63
		DSpC	No	No	Kruskal-Wallis w Dunn's	H = 1.694	0.61 (>0.99)	N/A
Phosphorylation	Ser98	VSpC	Yes	Yes - ↓ sALS vs Ct	Kruskal-Wallis w Dunn's	H = 8.018	**0.002 (0.0093)**	3.81
		DSpC	Yes	Yes - ↑ sALS vs Ct	Kruskal-Wallis w Dunn's	H = 9.018	**0.029 (0.021)**	2
	Ser102	VSpC	No	No	Kruskal-Wallis	H = 5.475	0.13	N/A
	Ser107	VSpC	No	No	Kruskal-Wallis	H = 0.2667	0.93	N/A

Succinylation	Lys9	VSpC	Yes	No	Kruskal-Wallis w Dunn's	H = 6.600	**0.05**	N/A
	Lys91	VSpC	No	No	Kruskal-Wallis	H = 0.5556	0.84	N/A
Ubiquitylation	Lys9	VSpC	No	No	Kruskal-Wallis	H = 5.284	0.15	N/A
	Lys91	VSpC	Yes	Yes - ↑ SOD1-*f*ALS vs Ct	ANOVA w Dunnet's	F = 8.944	**0.0008 (0.0027)**	2.53
		DSpC	No	No	ANOVA w Dunnet's	F = 1.371	0.28 (0.99)	N/A

Footnotes: [a] MMTS modifications to Cys residues signify reversible modifications to the residue *in vivo*, [b] NEM modifications to Cys residues indicate that the residue was unmodified *in vivo*.

Abbreviations: 3 DG-H, 3-deoxyglucosone-derived hydroimidazolone; AAAA, α-amino adipic acid; CEL, carboxyethyllysine; CML, carboxymethyllysine; Ct, control; DSpC, dorsal spinal cord; N/A, not applicable; PTM, post-translational modification; *SOD1*-fALS, *SOD1*-linked familial amyotrophic lateral sclerosis; sALS, sporadic amyotrophic lateral sclerosis; VSpC, ventral spinal cord.

Supplementary Table 9. Summary of alterations to SOD1 PTMs in the ventral spinal cord of familial (f)ALS and sporadic (s)ALS cases. Levels of histidine (His) and tryptophan (Trp) oxidation (Oxid.), Trp nitration (Nitr.), lysine (Lys) acetylation (Acet.), serine (Ser) phosphorylation (Phos.), glutamine (Gln) and asparagine (Asn) deamidation (Deam.), carboxyethyllysine (CEL), Lys ubiquitin footprint (Gly-Gly) and reversible (Revers.) modification of cysteine residues (Cys) varied significantly in individual ALS cases, and between ALS cases and controls. Arrows indicate significant increases or decreases in PTM levels compared with age-matched controls. Complete statistical test results are presented in **Supplementary Table 3**. Results from the quantification of SOD1 pathology (**Table 1**), SOD1 specific activity, and active SOD1 Cu:Zn and pI are included for comparisons. SOD1 pathology quantification classifications; + = >0-25%, ++ = 26-50%, +++ = 51-75%, ++++ = 76-100%.

Case #	Diagnostic Group	SOD1 Path.	SOD1 spec. act.	Active SOD1 Cu:Zn	Active SOD1 pI	Oxid. His48, His63	Oxid. Trp32	Nitr. Trp32	Acet. Lys3	Phos. Ser98	Deam. Gln15, Asn26, Asn53, Asn131	CEL Lys122, Lys128	Gly-Gly Lys91	Revers. Cys57, Cys146
11	fALS (SOD1, I113T)	++++	↓	↓	↑↑	↑	↑	ND	-	ND	↑	↑	↑	↑
12	fALS (SOD1, I113T)	++++	↓	-	↑↑	-	↑	-	ND	ND	↑	↑	↑	↑
13	fALS (SOD1, D101G)	++++	↓	↓	↑↑	↑	↑	-	-	ND	↑	↑	↑	↑
14	fALS (C9ORF72)	+	↓	↓	↑↑	↑	-	-	ND	-	-	ND	-	ND
15	fALS (unknown)	++	↓	-	↑	-	-	ND	-	-	-	-	-	-
16	fALS (C9ORF72)	+	↓	-	↑	-	-	ND	ND	ND	-	-	-	-
17	fALS (C9ORF72)	+	↓	NA	↑	-	-	ND	ND	ND	-	-	-	-
18	sALS	++++	↓	-	↑	-	-	↑	↓	ND	-	ND	-	↑
19	sALS	+	↓	NA	↑	-	-	↑	ND	↓	-	ND	-	ND
20	sALS	++	↓	-	↑	-	-	↑	↓	ND	-	-	ND	-
21	sALS	+	↓	NA	↑	-	-	ND	ND	ND	-	ND	-	-
22	sALS	+	↓	↓	↑↑	↑	-	ND	↓	↓	-	-	-	-
23	sALS	++	↓	-	↑	-	-	↑	↓	↓	-	-	-	-
24	sALS	+	↓	NA	↑	-	-	↑	↓	ND	-	-	-	-
25	sALS	++	↓	NA	↑	-	-	ND	ND	ND	-	ND	-	ND
26	sALS	NA	↓	↓	↑↑	↑	-	ND	↓	↓	-	-	-	-

Abbreviations: – ; no difference compared with controls; NA, tissue not available; ND, PTM not detected.

Supplementary Table 10. Characterization and quantification of spinal cord motor neuron (MN) CCS inclusions, as well as diffuse cytosolic and granular cytoplasmic CCS staining, in seven-micron paraffin-embedded formalin-fixed tissue sections of the cervical and thoracic spinal cord from all post-mortem tissue cases. Fixed tissues were not available (NA) for some cases. The four morphologies of CCS pathology were noted as present (√) or absent (-), and the proportion of motor neurons exhibiting any of the four morphologies quantified. The number of motor neurons exhibiting granular disordered CCS immunostaining was also quantified within each spinal cord ventral horn, expressed as a proportion of the total number of motor neurons in that ventral horn. Quantification classifications; - = 0%, + = >0-25%, ++ = 26-50%, +++ = 51-75%, ++++ = 76-100%.

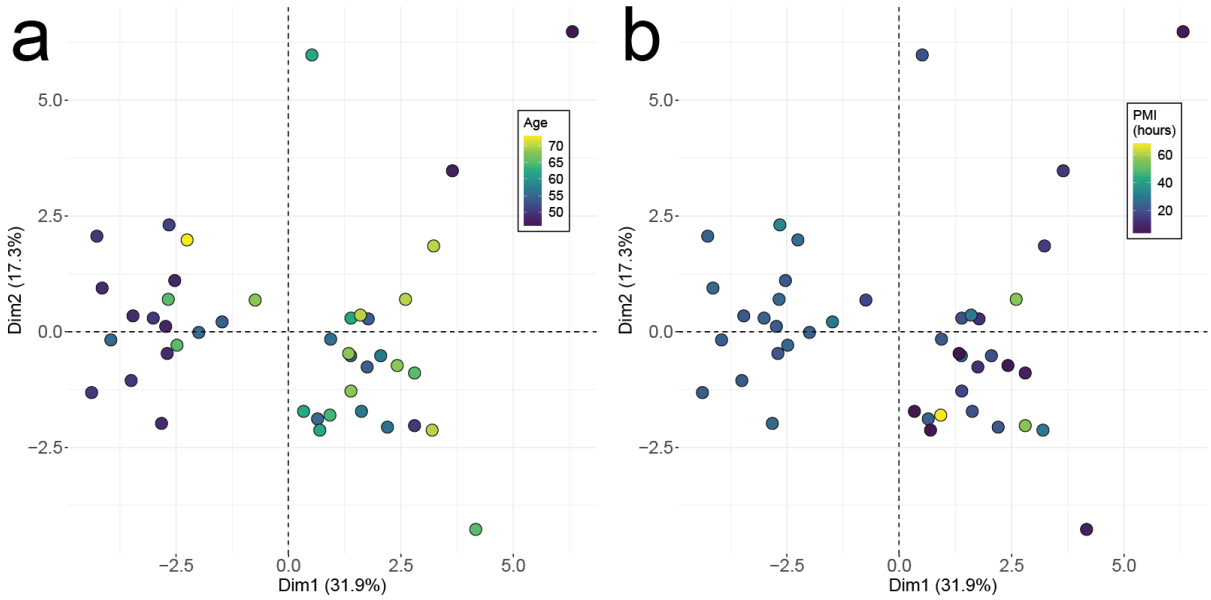
Case #	Diagnostic Group	CCS inclusions			Intense diffuse staining	Quant. (%MNs)	
		Globular	*Skein-like*	*Punctate*		*Inclusions + diffuse*	*Granular*
1	Age-matched Control	-	-	-	-	-	++
2	Age-matched Control	NA	NA	NA	NA	NA	NA
3	Age-matched Control	-	-	-	-	-	++
4	Age-matched Control	-	-	-	-	-	++
5	Age-matched Control	-	-	-	-	-	+++
6	Age-matched Control	-	-	-	√	+	++
7	Age-matched Control	-	-	-	√	+	++
8	Age-matched Control	-	-	-	√	+	++
9	Age-matched Control	-	-	-	√	+	++
10	Age-matched Control	-	-	-	√	+	++
11	fALS (SOD1, I113T)	√	-	√	√	++++	-
12	fALS (SOD1, I113T)	√	-	√	√	+++	-
13	fALS (SOD1, D101G)	√	-	-	√	+++	-
14	fALS (C9ORF72)	-	-	-	√	+	+
15	fALS (unknown)	-	√	-	√	++	+
16	fALS (C9ORF72)	-	-	-	√	++++	+
17	fALS (C9ORF72)	√	-	-	√	+	+
18	sALS	√	-	-	√	++++	+
19	sALS	-	-	√	√	++	-
20	sALS	-	-	-	-	-	+
21	sALS	NA	NA	NA	NA	NA	NA
22	sALS	-	-	-	√	+	+
23	sALS	-	-	√	√	-	+
24	sALS	-	-	-	√	+	+
25	sALS	-	-	-	√	+	+
26	sALS	NA	NA	NA	NA	NA	NA

Abbreviations: diff., diffuse; fALS, familial amyotrophic lateral sclerosis; Incl., inclusion; Quant., quantification; sALS, sporadic amyotrophic lateral sclerosis.

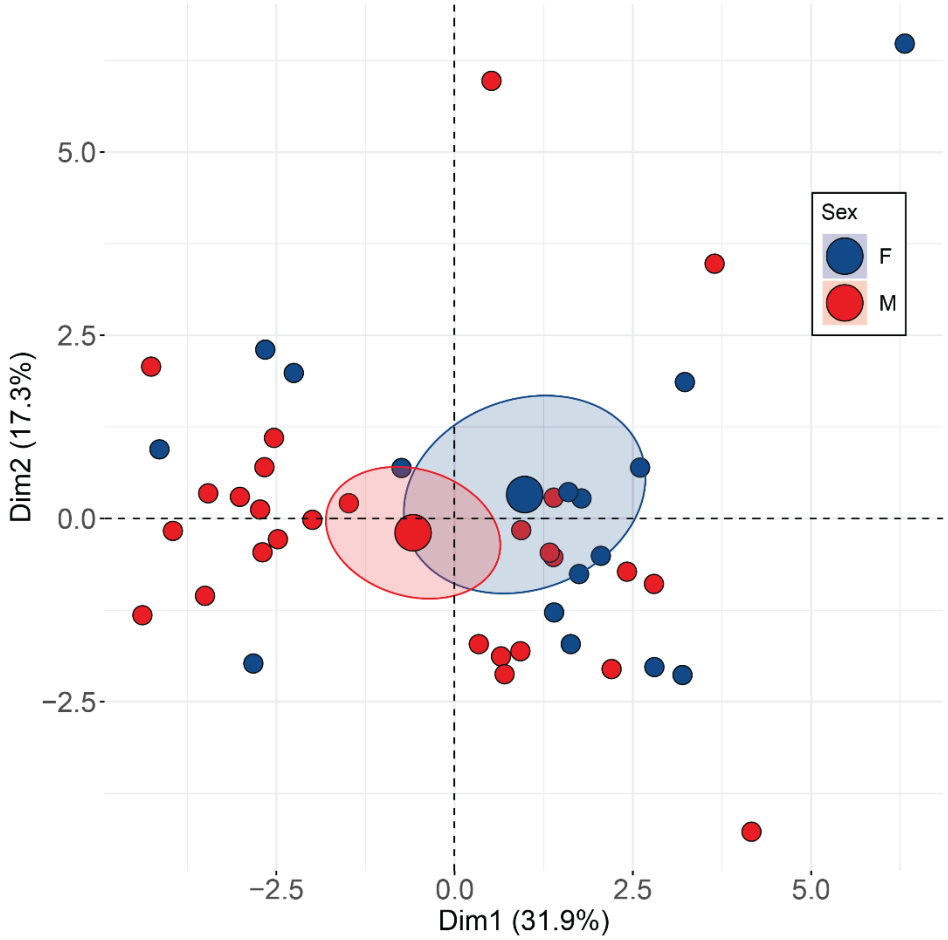
Supplementary Table 11. Complete details of statistical tests used to analyse differences in copper and zinc metal levels in the ventral and dorsal spinal cord between diagnostic groups in each tissue fraction. All comparisons were analysed using a Two-way ANOVA with Sidak's multiple comparisons post-hoc tests. ANOVA *p* values are listed in brackets following corresponding F statistics, whilst post-hoc *p* values reference comparisons drawn between ALS subgroups and controls, and are listed in brackets following their corresponding diagnostic group.

Biometal	Tissue fraction	Spinal cord subregion	Significant variation between groups?	ANOVA test statistics - F (p)	Significant differences between ALS subgroups vs controls (p)
Zn	Whole tissue	VSpC	Yes	25.42 (<0.0001)	Yes - ↑*SOD1*-fALS (<0.0001), non-*SOD1*-fALS (0.0001), sALS (<0.0001)
		DSpC			Yes - ↑non-*SOD1*-fALS (0.0186), sALS (<0.0001)
	Soluble	VSpC	Yes	11.05 (<0.0001)	Yes - ↑*SOD1*-fALS (0.0041), non-*SOD1*-fALS (0.021), sALS (<0.0001)
		DSpC			Yes - ↑sALS (0.016)
	Insoluble	VSpC	Yes	20.96 (<0.0001)	Yes - ↑*SOD1*-fALS (0.0003), non-*SOD1*-fALS (0.0094), sALS (<0.0001)
		DSpC			Yes - ↑non-*SOD1*-fALS (0.0149), sALS (0.0003)
Cu	Whole tissue	VSpC	No	1.086 (0.36)	No
		DSpC			No
	Soluble	VSpC	Yes	3.047 (0.034)	Yes - ↓*SOD1*-fALS (0.048), non-*SOD1*-fALS (0.011), sALS (0.043)
		DSpC			No
	Insoluble	VSpC	No	2.404 (0.074)	No
		DSpC			Yes - ↑sALS (0.043)

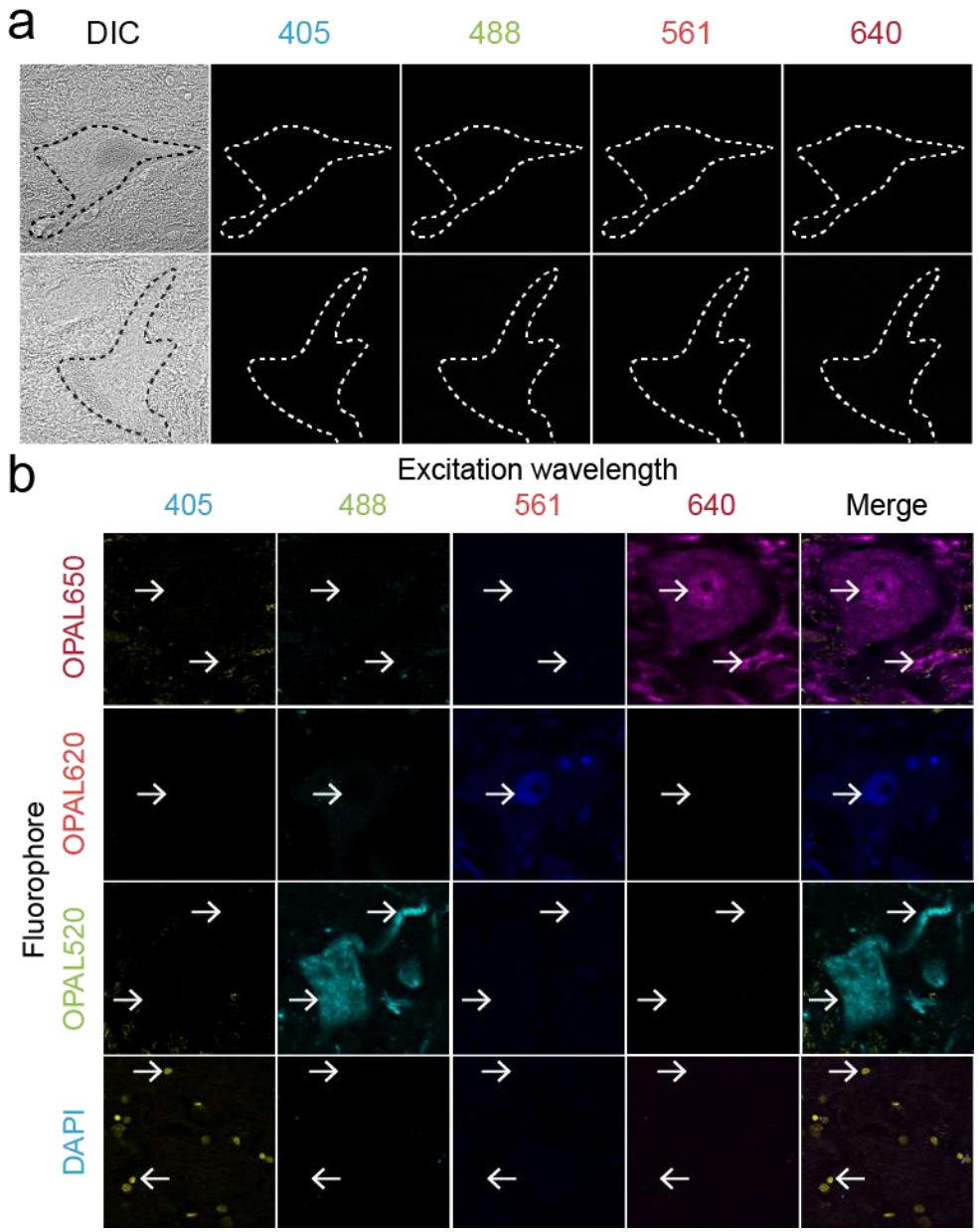
Abbreviations: DSpC, dorsal spinal cord; non-*SOD1*-fALS, non-*SOD1*-linked familial amyotrophic lateral sclerosis; sALS, sporadic amyotrophic lateral sclerosis; *SOD1*-fALS, *SOD1*-linked familial amyotrophic lateral sclerosis; VSpC, ventral spinal cord.



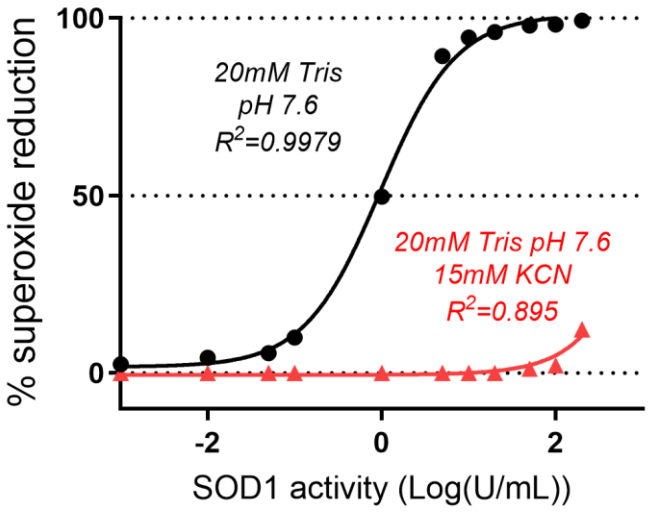
Supplementary Figure 1. A principal component analysis (PCA) did not identify any sample clustering by age or post mortem interval (PMI). a,b, PCA plots of cases coloured according to age (**a**) and PMI (**b**). Each point represents a ventral spinal cord sample (ALS and control). See methods for complete details of the variables included in this PCA.



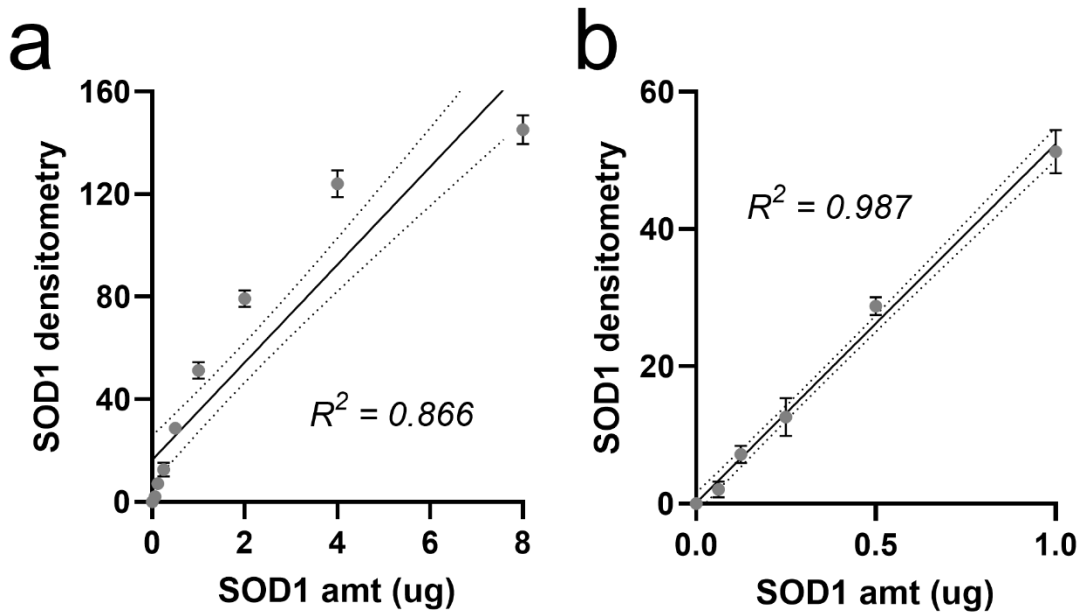
Supplementary Figure 2. A principal component analysis (PCA) found no sample clustering according to sex. PCA plot of individual ventral spinal cord samples from control or ALS cases. Blue dots represent samples obtained from females, and red dots are those from males. Large dots with coloured ellipses represent the centroid for that category or grouping and its 95% confidence intervals. See methods for complete details of the variables included in this PCA.



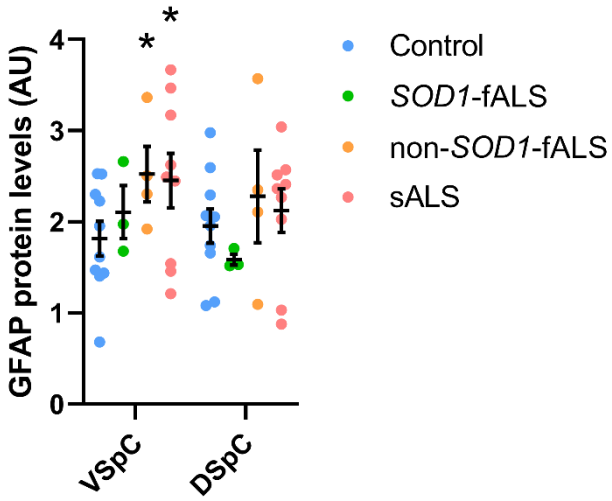
Supplementary Figure 3. **Validation of fluorescent microscopy workflow. a.** Characterisation of non-specific binding of secondary antibodies and OPAL fluorophores was performed using control tissue sections processed in the absence of primary antibodies. Negligible fluorescence was observed upon exposure of tissues to characteristic OPAL fluorophore excitation wavelengths, indicating minimal non-specific binding of secondary antibodies and OPAL fluorophores to tissues. **b.** To validate the absence of spectral overlap between our employed fluorophores at the confocal microscope acquisition settings employed in this study, tissue sections were labelled with TUJ-1 primary antibody (Biolegend, USA) and an anti-mouse HRP-conjugated secondary antibody, and were then labelled with individual OPAL fluorophores (OPAL650, OPAL620, OPAL520) or DAPI. Images were then captured at all four characteristic fluorophore wavelengths for each individually stained section using sequential acquisition, where fluorescent excitation and image acquisition are performed for each channel separately. This minimizes spectral bleed through between channels, as spectral emissions from one fluorophore cease prior to excitation and acquisition for the next channel.



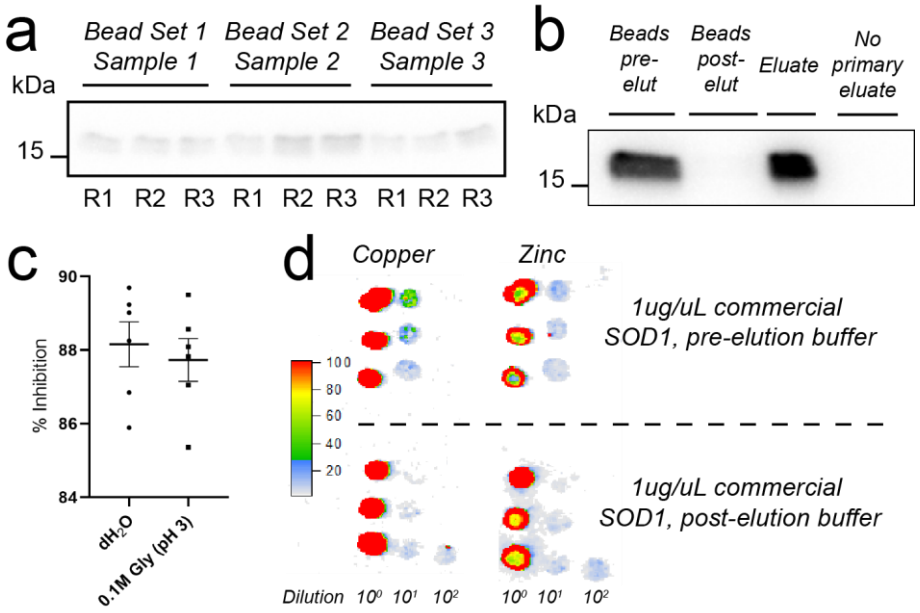
Supplementary Figure 4. Standard curve describing the relationship between % superoxide reduction and SOD1 activity under assay parameters. Curves were generated using commercial SOD1 (0.001-200U/mL, ≥2,500 units activity/mg protein; Sigma-Aldrich, USA) in the presence (red) and absence (black) of 15mM potassium cyanide (KCN). SOD1 activity values were transformed using X=Log(X) to improve non-linear regression curve fit.



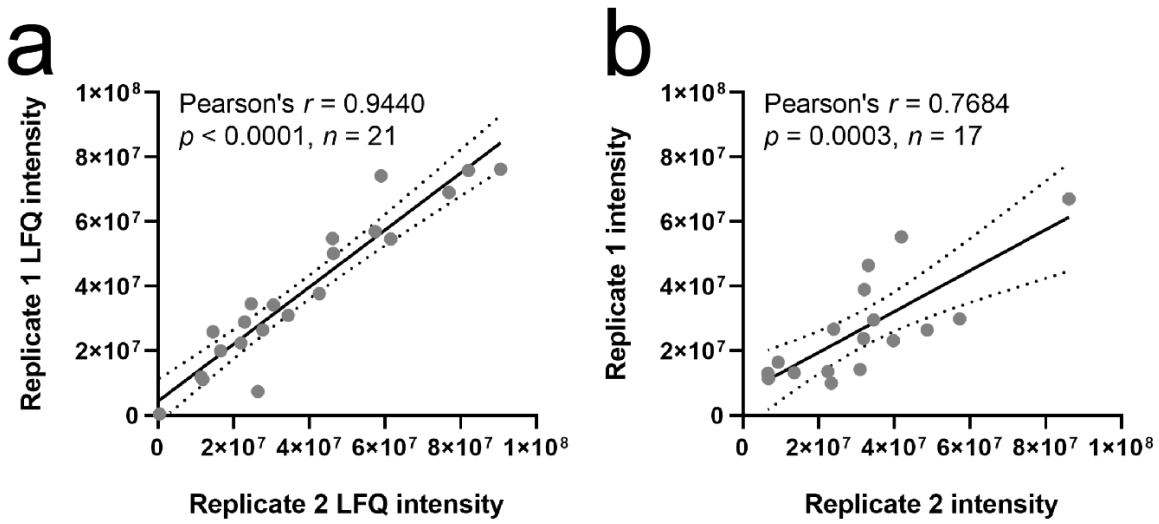
Supplementary Figure 5. Correlation between SOD1 protein levels and SOD1 immunoblot densitometry. Recombinant human SOD1 protein amount (ug) is linearly correlated with SOD1 immunoblot densitometry, as measured using ImageLab Software (Bio-Rad), when the amount of protein loaded per lane is less than 1ug. Data were obtained using triplicate sample preparations, and represent mean±SEM.



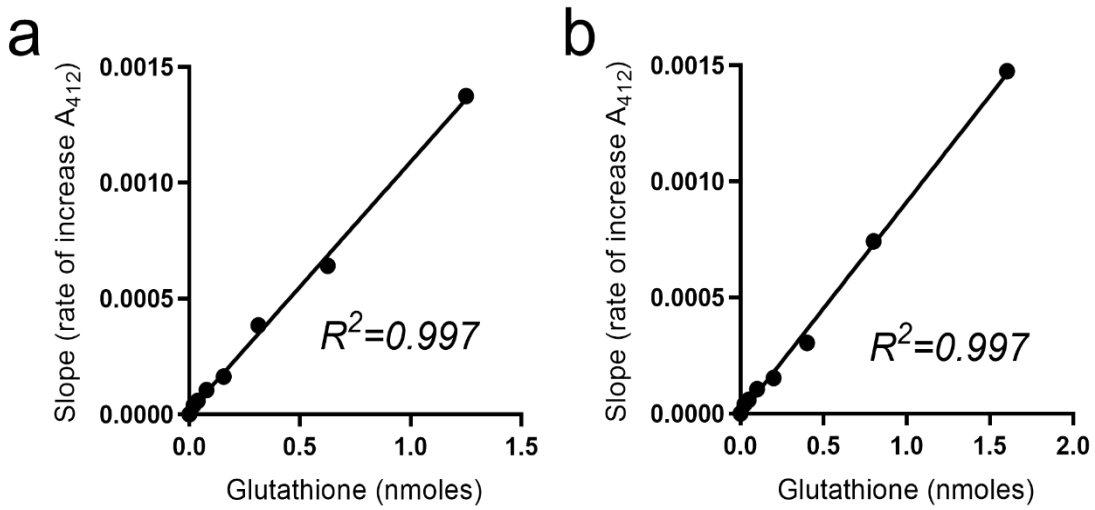
Supplementary Figure 6. GFAP protein levels in the ventral and dorsal spinal cord of all post-mortem cases. Protein levels were measured in the ventral (VSpC) and dorsal (DSpC) spinal cord using immunoblotting and are expressed as arbitrary units (AU). Two-way ANOVA with Sidak's multiple comparisons post-hoc tests revealed a significant increase in GFAP protein levels in the VSpC of non-*SOD1*-fALS and sALS cases compared with controls. *$p < 0.05$. Data represent mean±SEM.



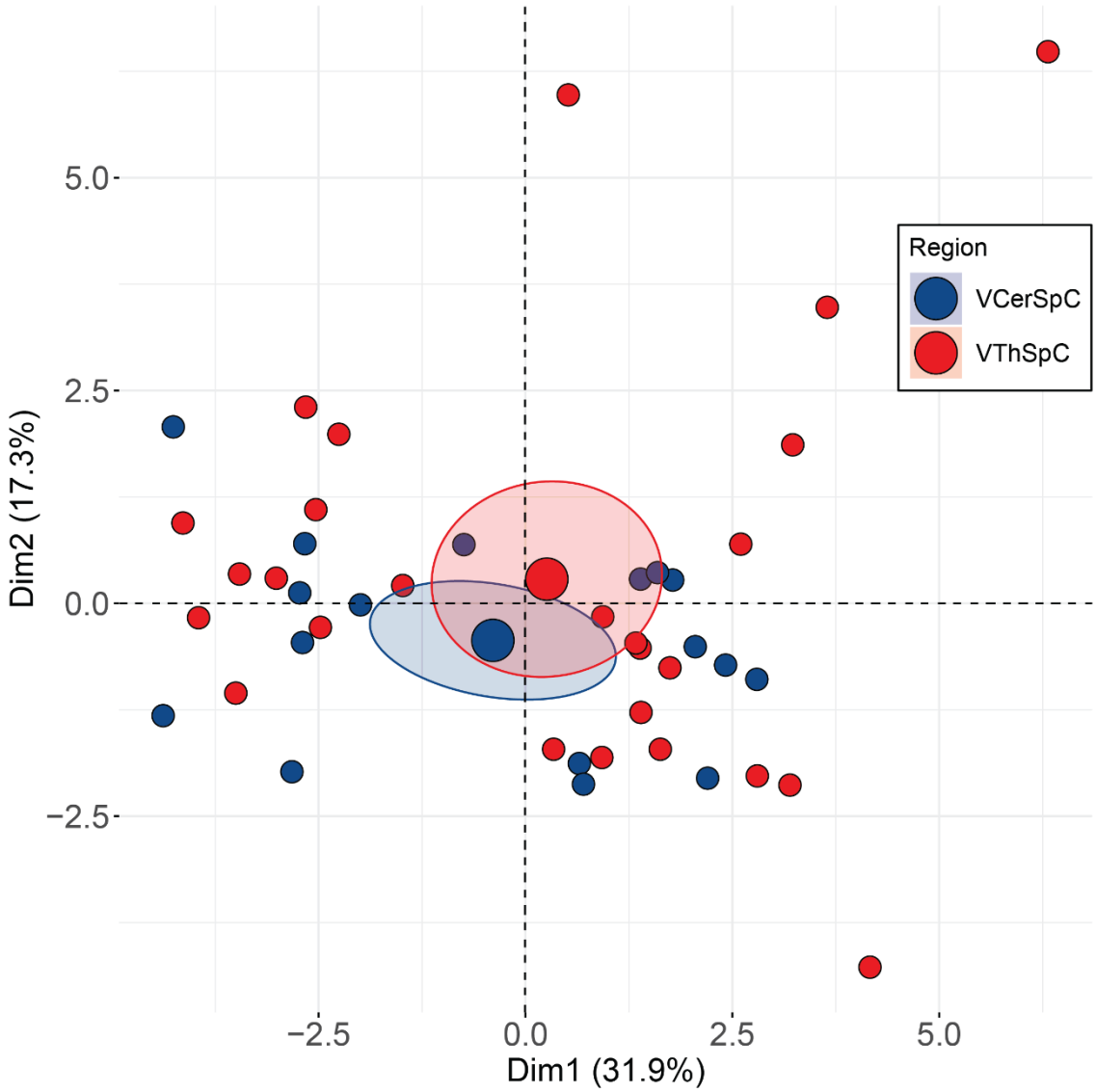
Supplementary Figure 7. Immunoprecipitation did not alter the metallation or antioxidant activity of a commercially available SOD1 standard. a, Immunoblotting of protein extracts, post-immunoprecipitation, for SOD1 revealed that 10mg of antibody-coupled dynabeads captured 90-95% of SOD1 protein from 200ug total protein extract. This capture efficiency did not diminish over three rounds (R1-3) of capture/elution using the same set of antibody-coupled dynabeads. **b,** 10mg antibody-coupled dynabeads were incubated with 200ug total protein extract to capture SOD1 protein. Beads were then divided in half, the second set underwent incubation with eluant, and eluate removed and stored separately for analysis. Both sets of dynabeads (pre- and post-elution) were then boiled in SDS-PAGE loading buffer, and the supernatants of these incubations, as well as the complete eluate resulting from elution of the second set of dynabeads, were immunoblotted for SOD1 protein. No SOD1 remained on dynabeads post-elution. SOD1 was also not identified in immunoprecipitates prepared using dynabeads that were not conjugated to our capture antibody (no primary). **c,d,** 10ug/uL of a commercially available SOD1 standard was diluted to 2ug/uL with 0.1M glycine (pH 3), and then further diluted to 1ug/uL with Ambic (pH 8), mimicking immunoprecipitation buffer conditions. The amount of SOD1 activity was measured in this solution, as well as in a 1ug/uL control solution of the SOD1 standard, and no difference in SOD1 activity was found between the two solutions (**c**; Mann-Whitney U test, $p = 0.59$, $U = 14$). The amount of copper and zinc bound to SOD1 was also measured in these solutions using a novel laser ablation-inductively coupled plasma-mass spectrometry protocol, and no difference in copper or zinc levels were observed (**d**; Mann-Whitney U test; copper: $p = 0.4$, $U = 2$; zinc: $p = 0.4$, $U = 2$).



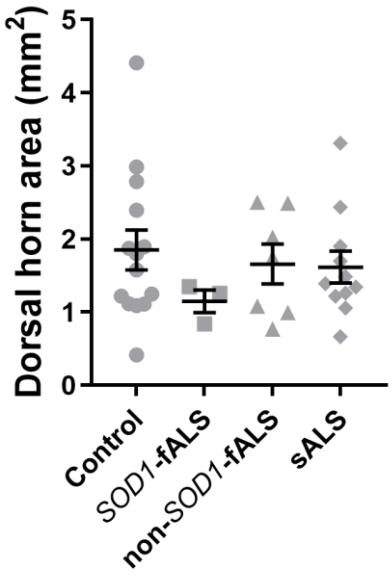
Supplementary Figure 8. The amount of SOD1 protein and its modified peptides are consistent between technical replicates analysed using liquid chromatography-tandem mass spectrometry. The amount of SOD1 protein (a) and redox modified histidine residue 46 (b) are highly correlated between technical replicates of the same sample, as determined by label-free quantification (LFQ) following liquid chromatography-tandem mass spectrometry (greater detail available in methods section). Pearson's correlation coefficient (*r*), the number of samples (*n*) and the statistical significance of each correlation (*p*) are included in each panel.



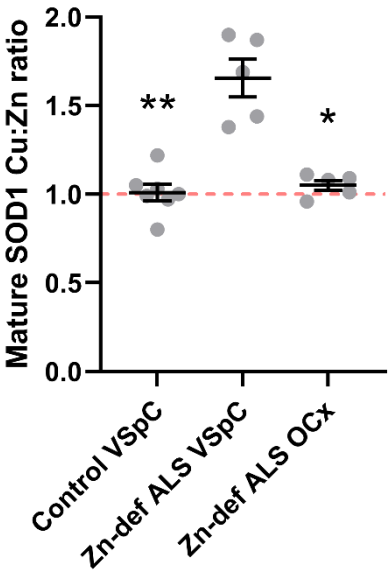
Supplementary Figure 9. Standard curves describing the relationship between the amount of glutathione and the rate of increase in absorbance at 412nm wavelength (A_{412}). a,b, Curves generated using commercial glutathione (0-1.6 nmoles) in the absence (**a**) and presence (**b**) of an adhesive plate seal used to maintain anaerobic conditions throughout the assay.



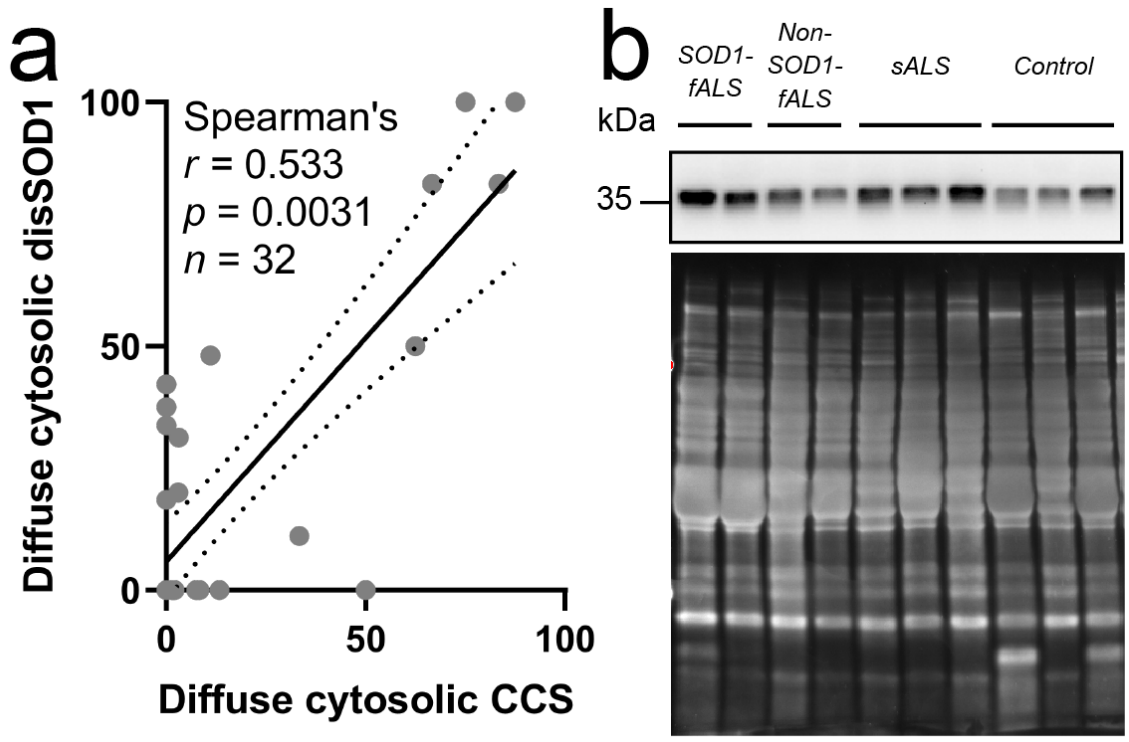
Supplementary Figure 10. A principal component analysis found no sample clustering according to ventral spinal cord level (cervical vs thoracic). PCA plot of ventral spinal cord samples from control and ALS cases. Blue dots represent samples obtained from ventral cervical spinal cord (VCerSpC), and red dots are those from ventral thoracic spinal cord (VThSpC). Large dots with coloured ellipses represent the centroid for that category or grouping and its 95% confidence interval. See methods for complete details of the variables included in this PCA.



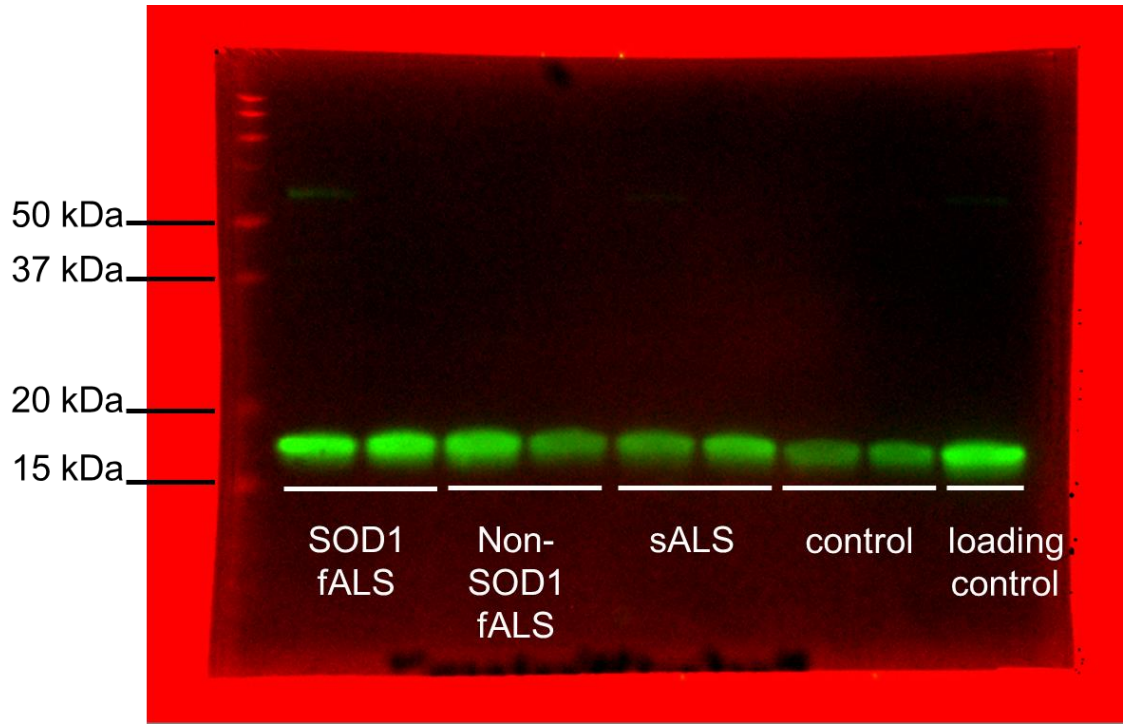
Supplementary Figure 11. Quantification of dorsal horn grey matter volume as an index of neurodegeneration in this region. Data represent mean ± SEM. Dorsal horn area did not differ between ALS subgroups and controls (Kruskal-Wallis H Test, $p = 0.7$, H = 1.42).



Supplementary Figure 12. Enzymatically-active, mature SOD1 copper:zinc ratio measured in the control and *SOD1*-fALS ventral spinal cord (VSpC) and the *SOD1*-fALS occipital cortex (OCx). Data represent mean ± SEM. The red dotted line marks the expected ratio of copper:zinc bound to enzymatically-active, mature SOD1. Active SOD1 copper:zinc ratios were elevated in the VSpC, but not OCx, of *SOD1*-fALS cases compared with controls (Kruskal-Wallis H test with Dunn's multiple comparisons post-hoc tests; *SOD1*-fALS VSpC: $p = 0.032$; *SOD1*-fALS OCx: $p = 0.59$). * $p < 0.05$ compared with the control VSpC.

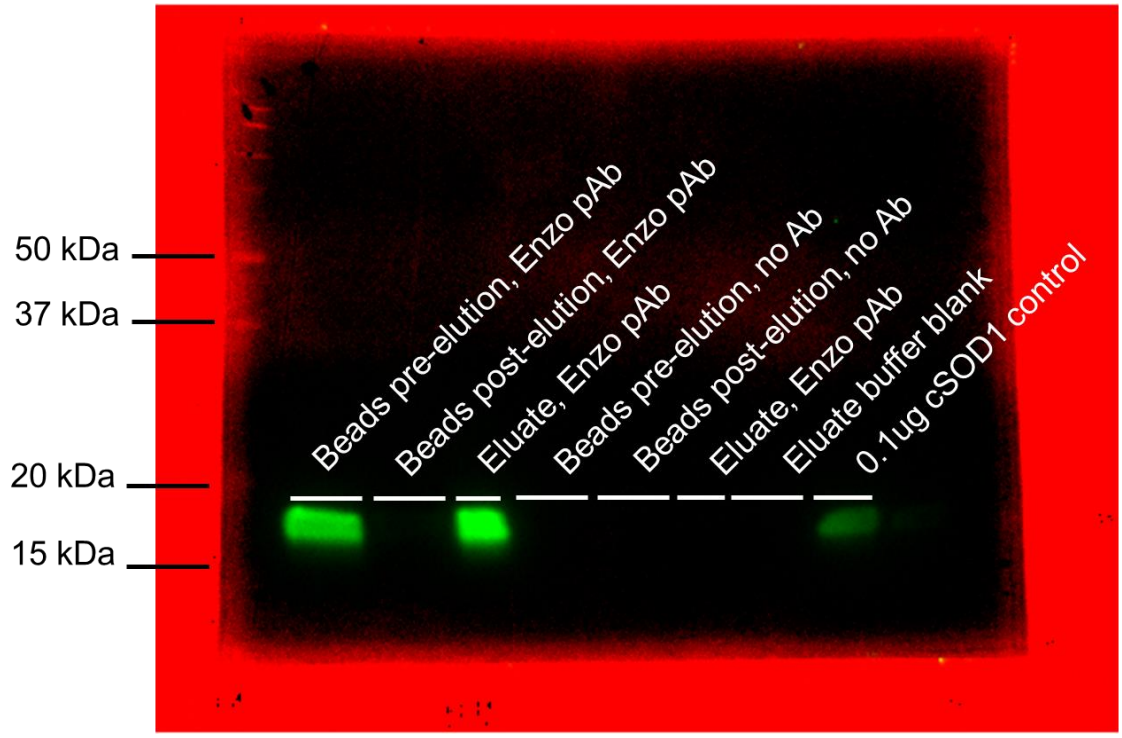


Supplementary Figure 13. Additional CCS protein data. a, Diffuse cytosolic CCS and disSOD1 are correlated in the ventral spinal cord. Spearman's r coefficient, p value and the number of XY pairs analysed (n) are stated within the panel. A correlation is strong if Spearman's r = 0.5 or higher. **b,** Representative CCS immunoblot and sypro ruby blot stain for total protein prepared from ventral spinal cord tissue extracts.

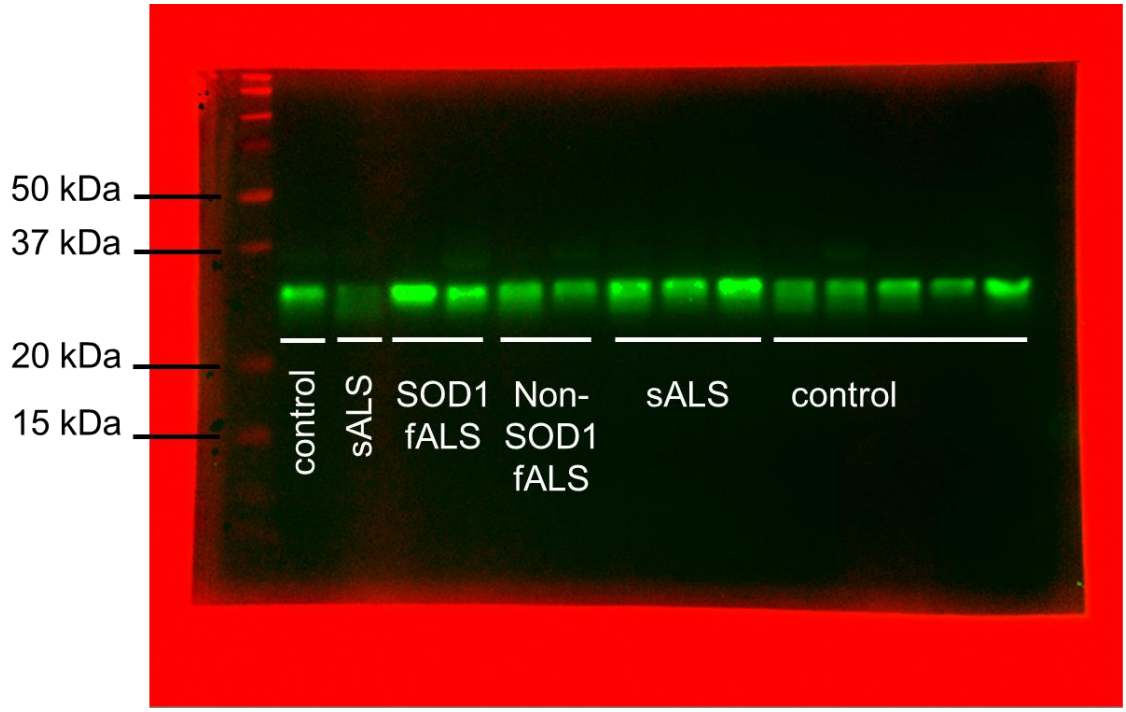


Supplementary Blot 1. Full-length blot used to make panel B of Figure 2. SOD1 chemiluminescence (Biolegend #850701) displayed in green, whilst Biorad Precision Plus Protein Dual Xtra Standards (Biorad #1610377) are displayed in red.

Supplementary Blot 2. Full-length blot used to make panel A of Supplementary Figure 7. SOD1 chemiluminescence (Biolegend #850701) displayed in black.



Supplementary Blot 3. Full-length blot used to make panel B of Supplementary Figure 7. SOD1 chemiluminescence (Biolegend #850701) displayed in green, whilst Biorad Precision Plus Protein Dual Xtra Standards (Biorad #1610377) are displayed in red. Abbreviations: cSOD1, commercial SOD1 standard (Sigma-Aldrich, USA).



Supplementary Blot 4. Full-length blot used to make panel B of Supplementary Figure 13. CCS chemiluminescence (Santa Cruz #sc-55561) displayed in green, whilst Biorad Precision Plus Protein Dual Xtra Standards (Biorad #1610377) are displayed in red.