Mutant SOD1 causes motor neuron disease independent of copper chaperone–mediated copper loading

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Copper-mediated oxidative damage is proposed to play a critical role in the pathogenesis of Cu/Zn superoxide dismutase (SOD1)–linked familial amyotrophic lateral sclerosis (FALS). We tested this hypothesis by ablating the gene encoding the copper chaperone for SOD1 (CCS) in a series of FALS-linked SOD1 mutant mice. Metabolic ^{64}Cu labeling in SOD1-mutant mice lacking the CCS showed that the incorporation of copper into mutant SOD1 was significantly diminished in the absence of CCS. Motor neurons in *CCS*$^{-/-}$ mice showed increased rate of death after facial nerve axotomy, a response documented for *SOD1*$^{-/-}$ mice. Thus, CCS is necessary for the efficient incorporation of copper into SOD1 in motor neurons. Although the absence of CCS led to a significant reduction in the amount of copper-loaded mutant SOD1, however, it did not modify the onset and progression of motor neuron disease in SOD1-mutant mice. Hence, CCS-dependent copper loading of mutant SOD1 plays no role in the pathogenesis of motor neuron disease in these mouse models.

Amyotrophic lateral sclerosis (ALS), a progressive, fatal neurodegenerative disease, is characterized by selective loss of motor neurons in the spinal cord, brain stem and motor cortex. The sporadic and familial forms of the disease have similar clinical and pathological features. Up to 10% of cases of ALS are familial (FALS), and ~25% of these are linked to mutation in Cu/Zn superoxide dismutase, an antioxidant enzyme that protects cells against superoxide toxicity[1,2]. A variety of studies, including investigations of SOD1-mutant mice that develop motor neuron disease[3–6], established that mutant SOD1 causes motor neuron disease through gain of one or more toxic properties[7,8]. However, the mechanism(s) whereby mutant SOD1 causes selective motor neuron death is not yet known. It has been speculated that cell death could be initiated by aberrant oxidative chemistry catalyzed by the copper atom bound in the mutant SOD1, especially after loss of zinc binding to SOD1[9]. Two prominent proposed oxidative chemistries are copper-dependent (i) peroxidation to generate hydroxyl radical[10] or (ii) nitration of tyrosines using peroxynitrite generated from the reaction of nitric oxide with superoxide[9,11].

In its free form, copper is toxic, and there is virtually no free copper in cells[12]. Normally, copper is imported into cells by a plasma membrane–bound copper transporter[13,14] and then shuttled to intracellular target proteins by a series of copper chaperones[15,16]. This is established for SOD1 based on the discovery in yeast of a copper chaperone (LYS7)[16] that delivers copper to SOD1 by direct protein–protein interaction[17]. Inactivation of the CCS gene in mice has shown[18] that CCS is required for the efficient incorporation of copper into SOD1 in mammals. We have taken advantage of this latter finding to determine whether the incorporation of copper into SOD1 is involved in the pathogenesis of SOD1 mutant–mediated motor neuron disease by generating a series of FALS-linked SOD1-mutant mice lacking CCS. We show here that SOD1-mutant mice lacking CCS have a selective defect in copper incorporation into wild-type and mutant SOD1. In addition, facial nerve axotomy studies in CCS-deficient mice indicated that CCS is necessary for efficient copper loading into SOD1 in motor neurons. Importantly, we show that the absence of CCS did not impact on the onset and progression of motor neuron disease in a series of FALS-linked SOD1-mutant mice. Taken together, our results demonstrate that mutant SOD1 induces motor neuron disease independent of CCS-mediated copper incorporation into mutant SOD1.

Results

Amounts of mutant SOD1 are independent of CCS

Mice with targeted disruption of one *CCS* allele (*CCS*$^{+/-}$ mice) were crossbred in two successive steps with mice expressing either wild-type or mutant (G93A, G37R or G85R) SOD1. This

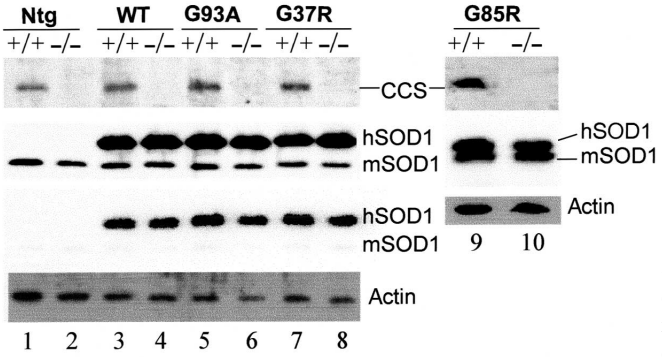


Fig. 1. SOD1 protein levels are not affected by the absence of CCS. Immunoblot analysis of spinal cord extracts from either normal mice, mice expressing human wild-type or mutant (G93A, G37R or G85R) SOD1 with or without CCS. Top, CCS protein levels. Middle, SOD1 protein levels using an antibody that recognizes human and mouse SOD1 with equal affinity. (Upper and lower middle, longer and shorter exposures of the same immunoblot, respectively.) Bottom, actin levels.

generated cohorts of littermates of either wild-type or mutant (G93A, G37R or G85R) SOD1 mice with or without CCS. As the amounts of mutant SOD1 present correlate with disease onset and survival[3,4], we first determined the amounts of mutant SOD1 in G37R, G93A or G85R SOD1 mice lacking CCS. Immunoblot analysis of spinal cord extracts showed that in $CCS^{-/-}$ mice the steady-state amounts of G37R, G93A or G85R SOD1 are similar to those in the respective $CCS^{+/+}$ mutant SOD1 mice (**Fig. 1**). Thus, the accumulation of mutant SOD1 in spinal cord and brain (data not shown) is independent of CCS.

Cu loading into mutant SOD1 is reduced without CCS

To confirm that the incorporation of copper into mutant SOD1 is dependent on CCS, we first determined the copper-dependent dismutase activity of mutant SOD1 from spinal cord extracts. In both the G37R and G93A mice, the enzyme activity from spinal cord lysates was markedly reduced in the absence of CCS as determined by an *in situ* gel assay[3] (**Fig. 2a,b**) or a solution assay[19] (**Fig. 2c**), despite the fact that amounts of mutant SOD1 are independent of CCS (**Fig. 1**). We did not detect any G85R-associated enzyme activity using both methods (**Fig. 2a,c**). To confirm that this reduction in enzymatic activity is due to a defect in copper incorporation into the catalytic domain, as we previously established for CCS deficient mice[18], and to determine whether copper is bound to any other region of mutant SOD1, we elected to perform ^{64}Cu metabolic labeling studies. After injection of ^{64}Cu into SOD1 mutant mice with or without CCS, we did not detect ^{64}Cu-labeled SOD1 in the spinal cords and brains of $G37R;CCS^{-/-}$ mice, whereas we detected robust ^{64}Cu-SOD1 labeling in $G37R;CCS^{+/+}$ and $G37R;CCS^{+/-}$ mice (**Fig. 3a,c**). As the amount of mutant SOD1 present is at least 10-fold higher than that of endogenous SOD1[3], and ^{64}Cu-labeled SOD1 can be readily observed in control mice[18], our failure to detect ^{64}Cu-labeled SOD1 in mutant SOD1 mice lacking CCS implies that there is at least a 90% reduction in the incorporation of copper into mutant SOD1. In addition, to assess copper homeostasis in mutant SOD1 mice lacking CCS, we examined both the overall copper content and copper trafficking to ceruloplasmin, a very sensitive indicator for aberrations in hepatic copper status. Copper loading studies of ceruloplasmin in G37R mutant mice lacking CCS showed no significant differences in copper incorporation into ceruloplasmin as compared to G37R mice with CCS (**Fig. 3c**). In addition, the oxidase activity of ceruloplasmin in the $G37R;CCS^{-/-}$ mice was similar to that in the $G37R;CCS^{+/+}$ mice (**Fig. 3c**). Atomic absorption spectrometric analysis showed no significant differences in copper content in the brain between mutant SOD1 mice with and without CCS (**Fig. 3d**). Thus, these results indicate that the absence of copper loading was selective for wild-type and mutant SOD1 and that copper homeostasis was normal in the $CCS^{-/-}$ animals.

Taken together, these results establish that CCS is necessary for the efficient incorporation of copper into mutant SOD1 in mice expressing ALS-linked SOD1 mutants. It still is possible, however, that the residual SOD1 activity (**Fig. 2a–c**) seen in SOD1-mutant mice lacking CCS is the result of selective, CCS-independent copper loading into SOD1 in motor neurons and that in tissue extracts containing the contents of several cell types, our methods are not sensitive enough to assess this magnitude

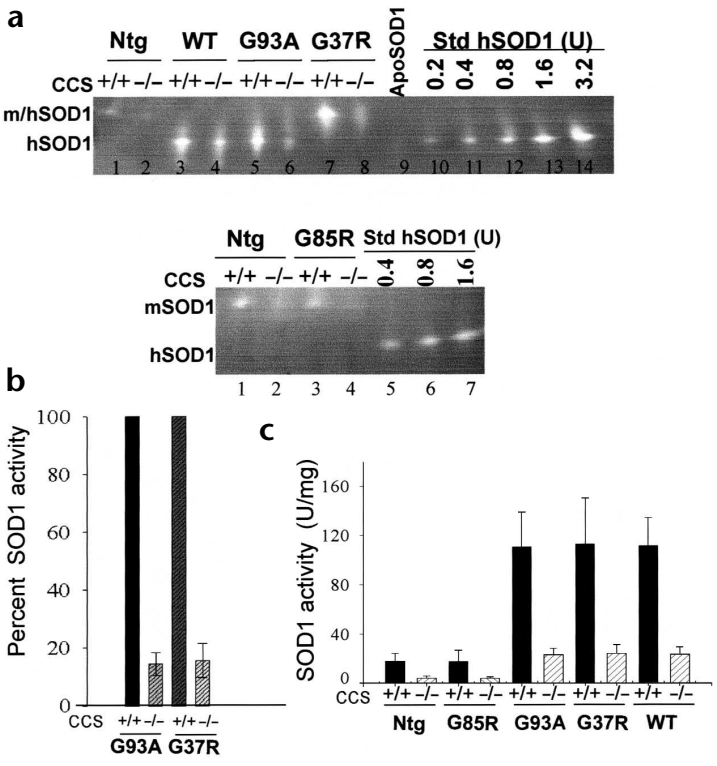


Fig. 2. Reduced SOD1 activity in mutant SOD1 expressing mice lacking CCS. (**a**) SOD1 activity gel of 30 μg protein extracts from spinal cords of normal (Ntg), human wildtype SOD1-overexpressing (WT), G37R and G93A mutant SOD1 mice with (+/+) or without CCS (–/–). Lanes 1 and 2, endogenous mouse SOD1 activity in the wild-type and CCS-null mice. Activity standards (Sigma; U, units of enzyme activity) are provided by different amounts of purified human SOD1 (lanes 10–14). Lane 9, apo-SOD1 (300 ng, without copper and zinc). Bottom, similar analysis as top, except for mice expressing G85R mutant SOD1. (**b**) CCS-dependent reduction in gel SOD1 activity assay in G93A (n = 3; reduction, ~84.5%) and G37R mice (n = 3; reduction, ~85%). (**c**) CCS-dependent reduction in solution SOD1 activity assay (n = 3) for non-transgenic mice, G85R, G93A, G37R and human wild-type SOD1-expressing mice.

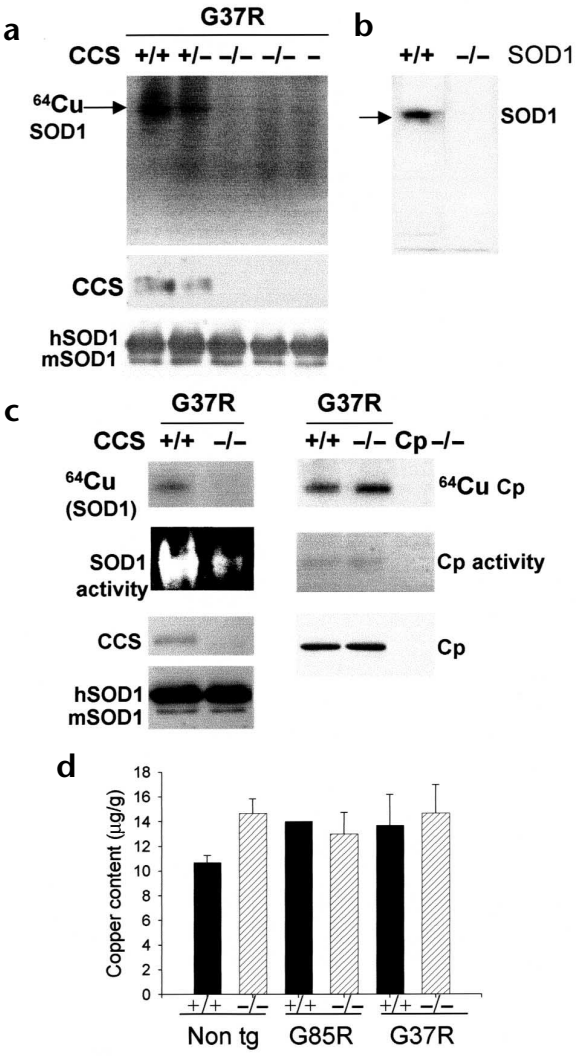


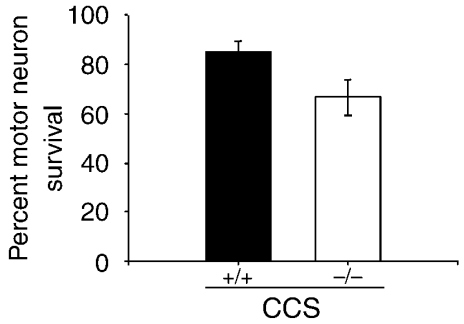
Fig. 3. Diminished ^{64}Cu incorporation into the mutant SOD1 protein in the absence of CCS. (**a**) Protein extracts of spinal cord were obtained from *CCS*$^{+/+}$;*G37R*, *CCS*$^{+/-}$;*G37R* and *CCS*$^{-/-}$;*G37R* mice after ^{64}Cu injection. Top, ^{64}Cu incorporation into G37R SOD1; middle, SDS-PAGE gel of spinal cord, immunoblotted with CCS antibody; bottom, same as middle, immunoblotted with human/mouse SOD1 antibody. (**b**) Specificity of SOD1 protein. Protein extracts of spinal cord from wild-type and SOD1$^{-/-}$ mice were resolved in a parallel native PAGE as in (**a**), followed by immunoblot analysis using h/mSOD1 antibody. (**c**) Normal copper incorporation and activity of ceruloplasmin (Cp) in the presence or absence of CCS. Spinal cord lysate of the G37R CCS$^{+/+}$ and CCS$^{-/-}$ mice were used. Left, ^{64}Cu incorporation in SOD1(top), a parallel G37R SOD1 activity native PAGE to show the specificity of SOD1 (top middle) and immunoblots probed for CCS (bottom middle) and human/mouse SOD1 (bottom). Right, ^{64}Cu incorporation into Cp of the plasma (top), activity of Cp (middle) and immunoblot for Cp protein levels (bottom) in G37R mice in the presence and absence of CCS or in mice lacking Cp (Cp$^{-/-}$) in the plasma. (**d**) Total copper content is unaltered in the absence of CCS in non-transgenic, mutant G37R/G85R brain tissue. Copper content (n = 3) is expressed as micrograms of copper per gram wet weight of brain tissue.

of SOD1 activity in these cells. As increased motor neuron death occurs after facial nerve axotomy in *SOD1*$^{-/-}$ mice relative to normal mice[20], a similar vulnerability to axotomy would be expected in *CCS*$^{-/-}$ mice if copper incorporation into SOD1 in motor neurons is deficient.

To test if CCS is necessary for SOD1 activity in motor neurons, unbiased stereology[21] was used to assess the magnitude of death after axotomy of motor neurons of the facial nuclei[22] in the brain stem of *CCS*$^{-/-}$ mice as compared to *CCS*$^{+/+}$ littermates. The *CCS*$^{-/-}$ mice showed an increased (~20%) loss of motor neurons (**Fig. 4**) compared to *CCS*$^{+/+}$ mice. This is the same magnitude of increased sensitivity to axotomy-induced motor neuron death observed in facial nuclei of *SOD1*$^{-/-}$ mice as compared to controls[20]. These results provide evidence that CCS is required for efficient incorporation of copper into SOD1 within motor neurons.

Mutant SOD1–induced disease proceeds without CCS

To determine the influence of CCS on the onset and progression of motor neuron disease in SOD1-mutant mice, we compared the time of disease onset and the length of survival of G37R, G93A or G85R SOD1 mice to these parameters in the respective SOD1-mutant mice lacking CCS. Onset of disease (**Table 1**) was not significantly different in the G37R or G93A SOD1-mutant mice lacking CCS as compared to the respective mutant SOD1 in either the *CCS*$^{+/+}$ or *CCS* $^{+/-}$ background (**Fig. 5a,b**). In addition, the mean survival time of the G37R, G93A or G85R mice (**Table 1**) was similar to that of the respective SOD1-mutant mice lacking CCS (**Fig. 5c–e**). Therefore, CCS-dependent copper loading has no impact on the onset and progression of motor neuron disease in SOD1-mutant mice.



To confirm that the SOD1-mutant mice lacking CCS showed the features of motor neuron disease seen in the SOD1-mutant mice, we carried out both clinical and neuropathological analysis of these cohorts of mice. Clinically, G37R, G93A or G85R mice lacking CCS were indistinguishable from the respective SOD1-mutant mice expressing CCS. Affected mice showed impaired extension of hind limbs when suspended by the tail, tremors, altered gait, coarse coat due to improper grooming, muscle wasting along the flanks, progressive slowness in the movement, and, near the end stage of the disease, inability to reach for food or turn over when placed on the back.

Neuropathologically, the SOD1-mutant mice with or without CCS in the late stage of disease showed severe degeneration and loss of motor neurons of the spinal cord (**Fig. 6a–d**). In mice

Fig. 4. Increased axotomy-induced death of motor neurons of facial nuclei in *CCS*$^{-/-}$ mice. Percent motor neuron survival is the ratio of surviving motor neurons of the axotomized to the number determined in the contralateral non-axotomized facial nucleus of *CCS*$^{+/+}$ (n = 7) and *CCS*$^{-/-}$ (n = 7) mice. There is ~20% reduction in neuronal survival after axotomy in the *CCS*$^{-/-}$ mice compared to normal mice (*CCS*$^{+/+}$, 84 ± 5%; *CCS*$^{-/-}$, 66 ± 7; $p < 0.001$; unpaired Student t-test). Error bars, s.e.m.

Table 1. Motor neuron disease onset and probability of survival of mutant SOD1 mice in the presence or absence of CCS.

Mutant SOD1	Onset of motor neuron disease (days)		Mean life span (days)		
	$CCS^{+/+}$ $CCS^{+/-}$	$CCS^{-/-}$	$CCS^{+/+}$	$CCS^{+/-}$	$CCS^{-/-}$
G37R	108 ± 13 (*n* = 4)	110 ± 0 (*n* = 3)	163 ± 17 (*n* = 10)	155 ± 15 (*n* = 15)	146 ± 15 (*n* = 11)
G93A	98 ± 10 (*n* = 5)	96 ± 6 (*n* = 2)	142 ± 13 (*n* = 13)	136 ± 10 (*n* = 33)	134 ± 9 (*n* = 10)
G85R	ND	ND	310 ± 45 (*n* = 9)	324 ± 66 (*n* = 13)	353 ± 66 (*n* = 10)

ND, not determined; *n*, number of mice.

expressing G37R or G93A SOD1, there was evidence of vacuolation of perikarya, axons and dendrites. When stained for SOD1 with an antibody recognizing mouse or human SOD1 with equal avidity, SOD1 was found in the cytoplasm surrounding the vacuoles (**Fig. 6g,h**). Motor neuron cell bodies, axons and dendrites also harbored aggregates of SOD1, ubiquitin and phosphorylated neurofilament protein (**Fig. 6g–l**). Within these spinal cord sections, reactive astrogliosis was detected on the basis of GFAP immunoreactivity (**Fig. 6m,n**). In the mutant G85R SOD1 mice lacking CCS, there was severe degeneration of motor neurons in the spinal cord (**Fig. 6d**) and aggregates containing SOD1, ubiquitin, phosphorylated neurofilament protein and reactive astrogliosis (data not shown).

In mutant mice with CCS, CCS was present in the motor neurons and astrocytes (data not shown) and occasionally in the neuropil, and CCS was seen in aggregates (**Fig. 6e**). As expected, in the $CCS^{-/-}$ spinal cord tissue, no CCS immunoreactivity was observed (**Fig. 6f**), but the absence of CCS did not abolish the other characteristic features of the neuropathology. At end stage of disease, SOD1, ubiquitin and phosphorylated neurofilament aggregates were observed in the spinal cord of all three lines of SOD1-mutant mice (G37R, G93A and G85R) irrespective of the presence or absence of CCS. Taken together, these results establish that CCS-mediated copper loading does not participate in the pathogenesis of motor neuron degeneration induced by mutant SOD1.

Discussion

Whether an oxidative mechanism involving copper underlies the pathogenesis of ALS linked to mutant SOD1 is still a subject of debate[23]. *In vitro* studies showing that either wild-type or mutant Zn-deficient SOD1 can cause peroxynitrite-mediated death of motor neurons in culture provide support for such a role of copper[9]. However, our findings demonstrating that a series of SOD1-mutant mice lacking CCS develop motor neuron disease provides compelling *in vivo* evidence that CCS-dependent copper loading onto SOD1 is not required for motor neuron disease mediated by mutant SOD1. It is also possible that such protective effect of blocking copper delivery to mutant enzymes may be offset by deleterious effects of as-yet-unidentified targets that may require CCS for copper loading. However, our demonstration that copper incorporation into ceruloplasmin is unaltered in SOD1-mutant mice lacking CCS (**Fig. 3c**) and our observation that CCS-null mice showed no abnormalities in copper uptake, distribution or loading into other cuproenzyme(s) leads us to believe it unlikely that other critical CCS-dependent targets exist.

Although it could be argued that the apparent (10–20%) residual SOD1 activity observed *in vitro* from analysis of spinal cord extracts of G37R or G93A SOD1 mice lacking CCS (**Fig. 2a,b**) reflects some CCS-independent copper incorporation, direct *in vivo* labeling with radioactive copper showed undetectable copper loading even onto mutant SOD1-G37R, which was previously reported to retain full dismutase activity[24]— although the failure to label the mutant enzyme might reflect a stable pool of holoSOD1 that is insensitive to copper exchange. In addition, previous studies in yeast have shown that G85R SOD1 (unlike other human SOD1 mutants) does not complement lysine auxotrophy in $sod1^{-/-}$, $lys7^{-/-}$ yeast cells[25], indicating that copper cannot be efficiently loaded

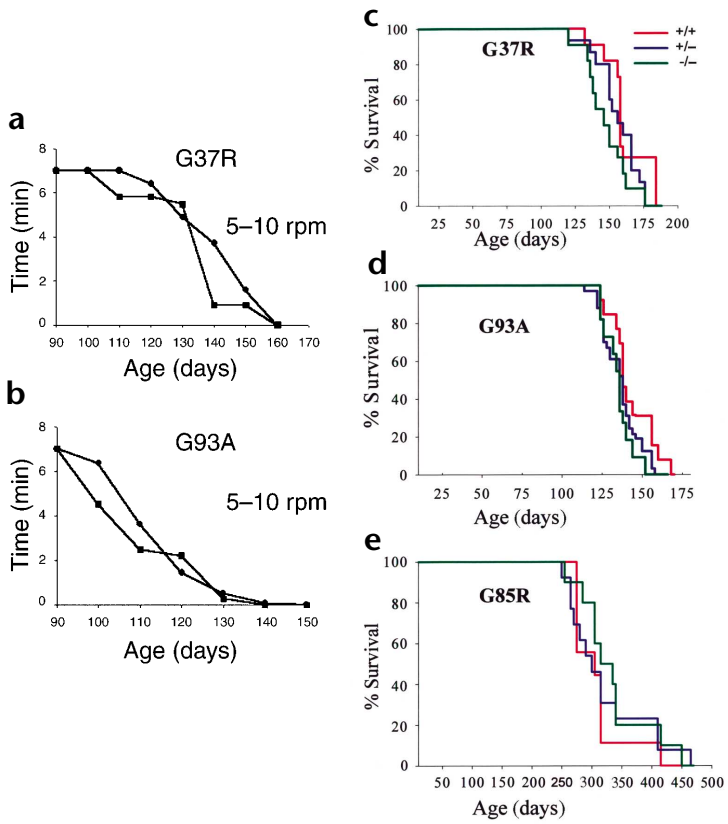
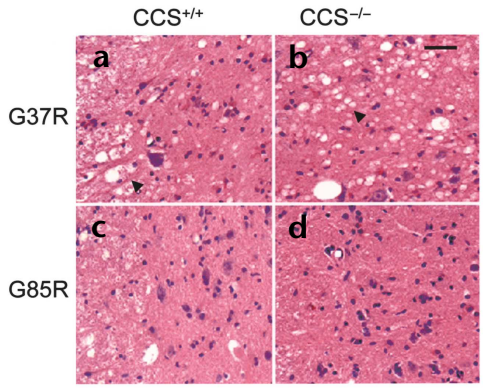


Fig. 5. Absence of CCS does not affect the onset of motor neuron disease or lifespan in G37R, G93A and G85R mutant mice. (**a**, **b**) Disease onset is not altered by the absence of CCS. The onset of disease is determined by motor function deficit seen in rotorod performance in G37R (**a**) and G93A (**b**) SOD1 mice in the presence (+/+ and +/–) and absence (–/–) of CCS. Motor functions were tested on the rotorod at an accelerated speed from 5 to10 rpm for 7 minutes. G37R mice, +/+ and +/– (*n* = 4; black circles); –/– (*n* = 3; black squares); G93A mice, +/+ and +/– (*n* = 5; black circles); –/– (*n* = 2; black squares). (**c**–**e**) The lifespan of mutant SOD1 mice is not altered by the absence of CCS. Kaplan–Meyer survival curves of G93A, G37R and G85R SOD1 mice in the presence (+/+ and +/–, red and blue, respectively) and absence (–/–, green) of CCS. G37R, +/+ (*n* = 10); +/– (*n* = 15); –/– (*n* = 11); G93A, +/+ (*n* = 13); +/– (*n* = 33); –/– (*n* = 10); G85R, +/+ (*n* = 9); +/– (*n* = 13); –/– (*n* = 10).



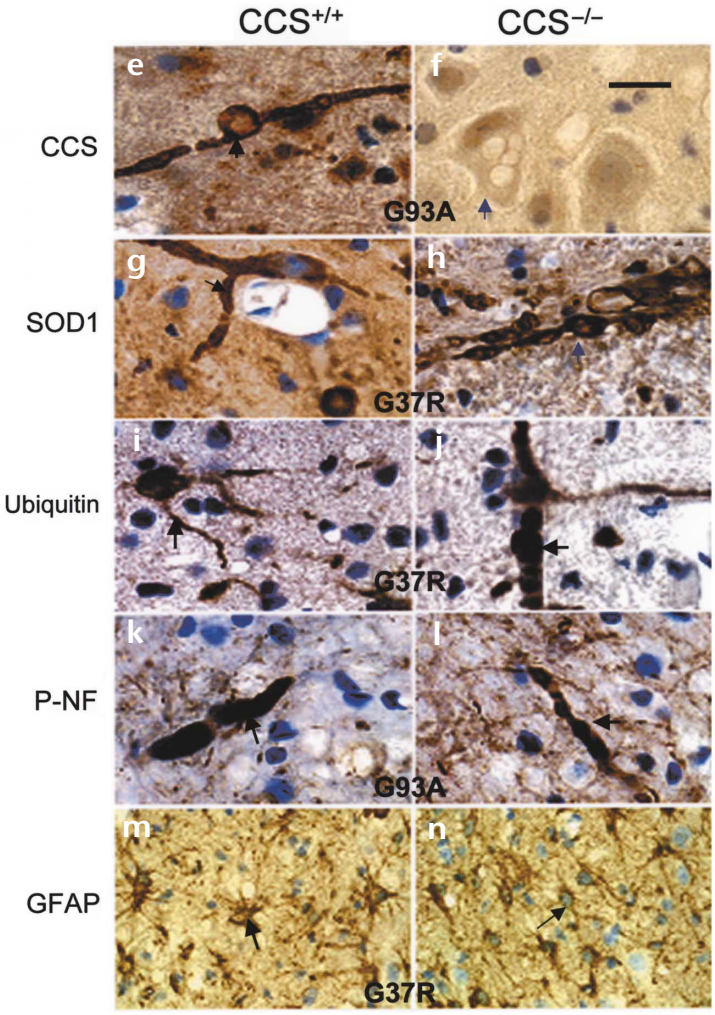


Fig. 6. Absence of CCS does not alter neuropathological abnormalities in end-stage G37R and G93A mutant SOD1 mice. Ventral horn of lumbar spinal cord sections stained with hematoxylin and eosin (**a**–**d**) revealed no gross differences in histological structures in G37R and G85R mice with or without CCS ($CCS^{+/+}$, $CCS^{-/-}$). Scale bar, 30 μm. Arrowhead, vacuoles in G37R mutant SOD1 mice. Immunocytochemical analysis of the ventral horn stained with antibodies to CCS (**e**, **f**), SOD1 (**g**, **h**), ubiquitin (**i**, **j**); phosphorylated neurofilament (P-NF; **k**, **l**); and GFAP (**m**, **n**). A surviving motor neuron with vacuoles is indicated by a blue arrow in (**f**). Astrogliosis was revealed by GFAP immunostaining (**m**, **n**). Scale bar, 12.5 μm.

onto the G85R SOD1 in the absence of CCS. Thus, given that the intracellular free copper level is very limiting[12], it seems most likely that part or all of the apparent *in vitro* activity of the G37R and G93A mutants in the absence of CCS may arise from copper acquisition by SOD1 during tissue homogenization or through another CCS-independent pathway. In addition, we found that motor neurons themselves were dependent *in vivo* on CCS for copper incorporation into SOD1, as facial motor neurons in $CCS^{-/-}$ mice had a similarly increased sensitivity to death after facial nerve injury as did those from the SOD1 deficient mice (**Fig. 4**). Even if we assume that there exists in the cell a CCS-independent pathway for loading copper onto SOD1 that may be responsible for the copper-mediated mutant SOD1 toxicity, CCS deletion in SOD1-mutant mice should favor an increase in CCS-independent copper loading. Our findings that disease onset and progression are not accelerated in SOD1-mutant mice lacking CCS are inconsistent with the view that such a CCS-independent copper loading pathway plays a crucial role in disease pathogenesis in the SOD1-mutant mice.

A strong prediction concerning the hypothesis of copper-mediated oxidative damage is that marked reduction of copper incorporation into SOD1 (to as little as 20% of normal) would significantly delay motor neuron disease onset or increase lifespan, or both. However, we did not see such an outcome. Even though *in vitro* cell culture studies have suggested that copper-mediated toxicity, particularly after loss of zinc from SOD1, plays a crucial role in disease induced by mutant SOD1[9,26], we found no differences in the clinical features of disease in the absence of CCS-dependent copper loading. Recent studies also provide evidence that is inconsistent with the possibility that peroxynitrite is a primary mediator of neurotoxicity in mutant SOD1[26–28]. Although initial studies[10,29,30] indicated that a copper-dependent mutant SOD1–induced peroxidase reaction could mediate toxicity, subsequent reports provide evidence against such a view[31,32]. It has also been proposed that copper bound to adventitious sites of mutant SOD1 could induce copper-mediated toxicity[33,34]. Although our findings documenting that significant diminution in copper loading to SOD1 did not ameliorate motor neuron disease in mutant SOD1 lacking CCS are inconsistent with the view that copper-dependent peroxidase–mediated toxicity plays a major role in the degeneration of motor neurons, they do not formally rule out such an hypothesis. Thus, we conclude that CCS-dependent aberrant copper chemistry is not involved in the pathogenesis of mutant SOD1–induced FALS.

Although the molecular mechanisms underlying mutant SOD1–linked FALS remain unclear, several pathogenic mechanisms other than the copper hypothesis[35–37] have been proposed. Mutant SOD1–containing aggregates have been implicated as participating in the pathogenesis of SOD1-linked FALS[38–40]. Although the direct role of these aggregates in disease pathogenesis is not yet clear, the identification of mutant SOD1-containing complexes as well as ubiquitin-positive inclusions as early changes in mutant SOD1 mice[38,39] supports the importance of mutant SOD1–associated aggregates in the pathogenesis of SOD1-linked FALS.

Methods

Mouse breeding. A two-step breeding strategy was employed to obtain mice without CCS. Initially, the mutant or wild-type (WT) human SOD1 mice were crossbred with $CCS^{+/-}$ mice to generate mutant or WT SOD1

with a single functional *CCS* allele. These mice were then crossbred with $CCS^{+/-}$ mice to generate mutant or WT SOD1 mice with two ($CCS^{+/+}$), one ($CCS^{+/-}$) or no ($CCS^{-/-}$) functional *CCS* alleles. All mice were housed at the Johns Hopkins University mouse facility under a 12 h light/12 h dark cycle. Food and water were provided *ad libitum*, and all care was given in compliance with National Institute of Health guidelines on the use of laboratory and experimental animals.

Determination of SOD1 activity. Spinal cord tissues from 2.5-month mice ($n = 2$ of each genotype) or terminally sick mice ($n = 2$) were homogenized in 20 mM Tris (pH 7), 1% Triton X-100, 1 mM EDTA, 1 mM bathocuproinedisulfonic acid (BCS) and protease inhibitor mixture (50 μg/ml leupeptin, 50 μg/ml pepstatin, 10 μg/ml aprotinin and 0.25 mM phenylmethylsulfonyl fluoride). After centrifugation at 10,000*g* for 10 min, 30 μg of the supernatant was fractionated by native polyacrylamide gel electrophoresis (PAGE) with 7.5% polyacrylamide and with 100 μM EDTA in the gel and the running buffer, and the SOD1 activity was determined using nitroblue tetrazolium reduction. The SOD1 activity was determined by densitometry. For immunoblot analysis, 2 μg of the same spinal cord tissue extracts in SDS sample buffer were separated on 16% SDS-PAGE, blotted to nitrocellulose membranes and probed with antibody against CCS or human or H/M SOD1 or antibody against actin.

Copper loading studies in mice. ^{64}Cu incorporation was carried out in 2.5-month mice by intraperitoneal and intracranial injection of 250 μCi of ^{64}Cu as described previously[18]. Twenty-four hours later, mice were anesthetized and plasma was harvested by collecting blood from tail-vein bleeds in EDTA and centrifuging it at 3,000*g* for 10 min at 4°C. Spinal cord tissue homogenate was prepared in 50 mM HEPES (pH 7.6), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA and a protease inhibitor mixture (Sigma, St. Louis, Missouri). Protein extracts (75 μg), obtained as supernatant after centrifugation at 25,000*g*, 10 min, 4°C, were separated by 10% native PAGE and exposed to PhosphorImager (Amersham, Sunnyvale, California) to detect holoSOD1 as described previously[16,18]. To detect ^{64}Cu labeling in ceruloplasmin, 2.5 μl of plasma was resolved by 4–20% native PAGE followed by exposure to PhosphorImager[18].

The oxidase activity of ceruloplasmin was determined by resolving 2.5 μl of plasma by 4–20% native PAGE and incubating the gels in 4.6 mM *p*-phenylenediamine/0.1 M sodium acetate, pH 5.2, at 37°C for 2 h in the dark[41]. Ceruloplasmin protein level was determined by immunoblot analysis with antisera to human ceruloplasmin.

Determination of copper content. Whole brains of 2.5-month nontransgenic and mutant SOD1 (G37R/G85R) mice with or without CCS were weighed and digested in concentrated nitric acid according to previously established methods[42] . Copper content was measured using a graphite furnace atomic absorption spectrophotometer (Perkin Elmer, Shelton, Connecticut).

Facial nerve axotomy. Mice (3 months old) were anesthetized with a mixture of oxygen, nitric oxide, and 2% enflurane (Abbott Labs, Abbott Park, Illinois) through a specially designed nose adapter, and were operated on under sterile conditions. The facial nerve was transected just distal to the exit from the stylomastoid foramen and the proximal stump was ligated to prevent regeneration. After 5 weeks, the mice were deeply anesthetized with chloral hydrate and perfused with cold phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed for 2–3 days and cryoprotected; then 40-μm-thick serial sections were cut on a freezing microtome and stained with cresyl violet. Total number of motor neurons in the facial nuclei was counted using unbiased stereological methods.

Determination of disease onset and progression. Onset time was assigned as the age at which motor deficits were manifested as the inability of the mice to remain on the rotorod (Columbus Instruments, Columbus, Ohio) for 7 min at different speeds (5–10 rpm, 10–15 rpm, and 15–20 rpm). Initially, the mice were trained for a total of ~30 min on the rotorod at the three speeds (7–10 min per speed) every day for 5 days. Performance was then determined at intervals of 1 week or 3–4 days throughout disease progression. Terminal illness was defined by either the inability of a mouse to right itself in 10 sec when left on its back or the inability to reach for food or water.

Histology and immunohistochemistry. Terminally sick mice were perfused with PBS followed by 4% paraformaldehyde in PBS. The spinal cord was removed, embedded in paraffin, sectioned (10 μm), deparaffinized using standard protocols and stained with hematoxylin and eosin or with specific antibodies.

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