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Biological effects of CCS in the absence of SOD1 enzyme activation: implications for disease in a mouse model for ALS

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The CCS copper chaperone is critical for maturation of Cu, Zn-superoxide dismutase (SOD1) through insertion of the copper co-factor and oxidization of an intra-subunit disulfide. The disulfide helps stabilize the SOD1 polypeptide, which can be particularly important in cases of amyotrophic lateral sclerosis (ALS) linked to misfolding of mutant SOD1. Surprisingly, however, over-expressed CCS was recently shown to greatly accelerate disease in a G93A SOD1 mouse model for ALS. Herein we show that disease in these G93A/CCS mice correlates with incomplete oxidation of the SOD1 disulfide. In the brain and spinal cord, CCS over-expression failed to enhance oxidation of the G93A SOD1 disulfide and if anything, effected some accumulation of disulfide-reduced SOD1. This effect was mirrored in culture with a C244,246S mutant of CCS that has the capacity to interact with SOD1 but can neither insert copper nor oxidize the disulfide. In spite of disulfide effects, there was no evidence for increased SOD1 aggregation. If anything, CCS over-expression prevented SOD1 misfolding in culture as monitored by detergent insolubility. This protection against SOD1 misfolding does not require SOD1 enzyme activation as the same effect was obtained with the C244,246S allele of CCS. In the G93A SOD1 mouse, CCS over-expression was likewise associated with a lack of obvious SOD1 misfolding marked by detergent insolubility. CCS over-expression accelerates SOD1-linked disease without the hallmarks of misfolding and aggregation seen in other mutant SOD1 models. These studies are the first to indicate biological effects of CCS in the absence of SOD1 enzymatic activation.

INTRODUCTION

Eukaryotic Cu, Zn-superoxide dismutase (SOD1) plays an important cellular role in antioxidant defense through its ability to scavenge superoxide anion using copper redox chemistry (1). SOD1 mainly acquires its catalytic copper co-factor by direct copper transfer from its copper chaperone protein, CCS (2). Another post-translational modification critical to the function of SOD1 is oxidation of an intra-subunit disulfide bond. CCS promotes oxidation of the SOD1 disulfide in an oxygen-dependent process that is proposed to proceed through transfer of a disulfide that first forms between CCS and SOD1 (3–5). In the case of human SOD1, some activation of the enzyme through disulfide oxidation and copper insertion can also be achieved through a CCS-independent pathway, currently of unknown nature (4,6). Copper acquisition and disulfide oxidation are not only critical for enzyme activity, but have long been known to play an important role in the structural stability of SOD1 (7).

Although SOD1 normally protects cells against oxidative stress, dominant mutations spread throughout the SOD1 polypeptide are linked to familial amyotrophic lateral sclerosis (ALS). The mechanism through which mutant SOD1s cause this fatal neurodegenerative disease is not clear, but a prominent hypothesis involves instability, misfolding and aggregation of mutant SOD1 (8–10). The histological appearance of proteinacious inclusions in the spinal cord is one hallmark of

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disease (8,11,12), and as early indicators of disease, biochemical markers of SOD1 misfolding have been observed. One prominent biochemical marker is the appearance of detergent insoluble precipitates of SOD1. These precipitates have been noted with a wide array of SOD1 mutants and correlate well with disease onset and progression (13–23).

Multiple lines of study have implicated loss of the SOD1 intra-molecular disulfide in misfolding of SOD1 (24–29). Since CCS promotes oxidation of the SOD1 disulfide, one might expect the copper chaperone to be beneficial in preventing misfolding and aggregation of mutant SOD1. Yet in a recent study, CCS over-expression was found to greatly accelerate disease in a mouse model for SOD1-linked ALS (30). Mean survival for mice expressing G93A SOD1 shifted from 242 days to 36 days in G93A SOD1 mice over-expressing CCS (referred throughout as G93A/CCS mice) (30). G93A/CCS mice also displayed spasticity and hyperreflexia (30), symptoms consistent with marked upper motor neuron pathology. The finding that disease is accelerated by CCS over-expression would seem to argue against a role for disulfide oxidation in helping to promote mutant SOD1 stability and prevent disease. Yet it was not clear whether CCS was actually enhancing disulfide oxidation in this mouse model.

Here we employed a biochemical approach to better understand the impact of CCS over-expression on the SOD1 disulfide and misfolding of the polypeptide. We find that as expected, CCS over-expression promotes oxidation of a wild-type (WT) SOD1 disulfide. However, in the diseased G93A/CCS mouse, there was no increased oxidation of the mutant SOD1 disulfide; if anything, CCS over-expression correlated with some increase in disulfide reduction. Moreover, there was no biochemical evidence of SOD1 misfolding in the diseased G93A/CCS mouse as monitored by formation of detergent insoluble precipitates of SOD1, consistent with the previously observed lack of detectable SOD1 inclusions in the spinal cord of these mice (30). This effect of CCS over-expression, i.e. disulfide reduction without an increase in aggregation, was also obtained in cell culture using an inactive allele of CCS. Hence, aberrant effects of CCS on the SOD1 disulfide can be on the pathway to disease through a mechanism that need not involve mutant SOD1 aggregation.

RESULTS

The effects of CCS over-expression on mutant SOD1 expressed in mice

Since CCS plays a role in oxidation of the SOD1 disulfide (4,5) we probed the status of the SOD1 disulfide in mice over-expressing CCS, particularly in the diseased G93A/CCS mice. We employed two independent detection assays: the AMS assay, which involves covalent modification of reduced cysteines with the large thiol-reactive maleimide AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) (adapted for tissue from 4) and the iodoacetamide (IAM) assay recently developed by Jonsson *et al*., where disulfide-oxidized and disulfide-reduced SOD1 are differentiated by non-reducing gel electrophoresis following thiol protection by IAM (29,31).

The mice chosen for these studies included 20–49-day-old non-transgenic (ntg), CCS transgenic (CCS), WT SOD1 transgenic (WT SOD1, line N29), G93A transgenic (G93A; Gur^{dl} line) and both the WT SOD1/CCS and G93A/CCS double transgenic crosses (from either line 17 or line 21 crosses as indicated) (30). G93A/CCS double transgenic mice were symptomatic at the ages analyzed, while all other mice were disease free (30). The SOD1 disulfide was analyzed in the brain as well as in the spinal cord. Using the IAM method we observed that in the brains of both WT and G93A-mutant SOD1 mice the SOD1 disulfide is largely oxidized (note: each lane represents a separate mouse), and the percentages of disulfide-reduced in the mice analyzed ($\approx$14% for WT and $\approx$8–9% for G93A; Fig. 1A, lanes 3, 5 and 7) are comparable to values previously reported for older transgenic mice over-expressing WT or G93A SOD1 (13 and 5%, respectively) (31). When CCS is over-expressed in WT SOD1 transgenic mice, the WT SOD1 disulfide is even more oxidized (Fig. 1A, lane 4, Fig. 1B). This same result was observed with independent mice analyzed by the AMS assay for the disulfide (Supplementary Material, Fig S1A). The increase in disulfide oxidation is expected, given the predicted role for CCS in oxidizing the SOD1 disulfide (5,6,10). However, in symptomatic G93A/CCS mice, there is no increase in oxidation of the mutant SOD1 disulfide. Instead, the opposite trend was observed and the disulfide was somewhat more reduced (Fig. 1A, lanes 6 and 8; Supplementary Material, Fig. S1A). Note that this effect is similar in two distinct lines of G93A/CCS mice: one with a mean survival of 36 days, produced from a cross with line 17 CCS transgenic mice (lane 6); and one with a mean survival of 56 days, produced from line 21 CCS transgenic mice with somewhat lower levels of CCS expression (Fig. 1A, lane 8) (30). The same results on increased disulfide reduction were evident with independent mice analyzed by the AMS assay (Supplementary Material, Fig. S1A). We also observed that the proportion of the G93A SOD1 polypeptide that was enzymatically active did not increase with CCS over-expression (Supplementary Material, Fig. S2), consistent with the observed lack of increased disulfide oxidation and perhaps also copper loading. Quantitation of the IAM and AMS analyses revealed that both the percentage of disulfide-reduced G93A SOD1 (Fig. 1A, lanes 6 and 8; Supplementary Material, Fig. S1A), and the total level of disulfide-reduced G93A SOD1 normalized to an alpha-tubulin control (Fig. 1B) were increased with CCS over-expression.

Similar effects on the disulfide were observed in spinal cord. As seen in Figure 2A, the disulfide of WT and G93A SOD1 is largely oxidized (lanes 2–3, 6–8), and the percentages of disulfide-reduced SOD1 from the individual mice analyzed ($\approx$15–17% for WT and $\approx$12–13% for G93A) are similar to a previous report by Jonsson *et al*. (14 and 8%, respectively) in 100-day-old WT SOD1 and G93A mice (31). CCS over-expression is again associated with an increase in disulfide oxidation for WT SOD1 in spinal cord (Fig. 2A, lanes 4 and 5) and the same results were observed in an independent mouse analyzed by AMS (Supplementary Material, Fig. S1B). However, in all four symptomatic G93A/CCS mice analyzed (Fig. 2 and Supplementary Material,

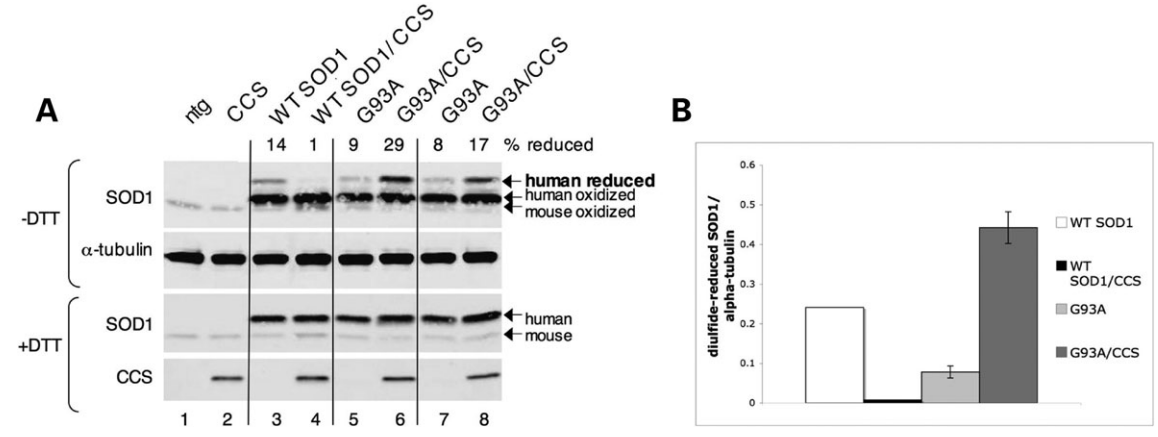

Figure 1. SOD1 disulfide analysis in brain. (**A**) Brain samples were prepared from the indicated mice in the presence of iodoacetamide (IAM) as described in Materials and Methods. Three micrograms total protein from each individual brain were treated either with (+DTT) or without (−DTT) reducing agents prior to SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting using a polyclonal antibody that recognizes both mouse and human SOD1 and Alexa Fluor goat anti-rabbit 680 secondary antibody. Where indicated, blots were re-probed with antibodies against chicken alpha-tubulin (Sigma T6199) and mouse CCS. Quantitation of disulfide-reduced and disulfide-oxidized human SOD1, normalized against alpha-tubulin, was carried out with the LI-COR Odyssey software as described in Materials and Methods. Numbers at top indicate the percentage of total human SOD1 that is disulfide-reduced for each individual mouse analyzed. (**B**) The total level of disulfide-reduced SOD1 normalized to alpha-tubulin was averaged over the mice analyzed in part (A) and plotted. Error bars represent range. The individual mice analyzed included: ntg, 38 day-old non-transgenic mouse; CCS, 33-day-old CCS (line 17 (30)) mouse; WT SOD1, 48-day-old WT SOD1 mouse; WT SOD1/CCS, 48-day-old WT SOD1/CCS (line 17) dual transgenic mouse; G93A (lane 5), 38-day-old G93A mouse; G93A/CCS (lane 6), 33-day-old G93A /CCS (line 17) dual transgenic mouse; G93A (lane 7), 46-day-old G93A mouse; G93A/CCS (lane 8), 46-day-old G93A/CCS (line 21 (30)) dual transgenic mouse.

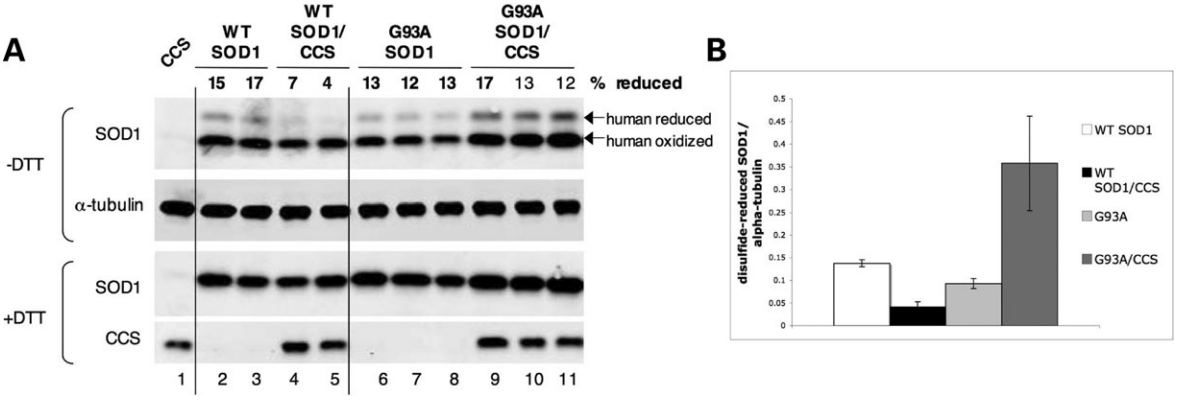

Figure 2. SOD1 disulfide analysis in spinal cord. The SOD1 disulfide was analyzed as in Figure 1 using 1.5 μg protein samples from individual spinal cords prepared in the presence of IAM. (**A**) Immunoblot as in Figure 1A, but with human-specific SOD1 antibody; (**B**) Quantitation of disulfide-reduced SOD1 as in Figure 1B. The individual mice included: CCS, 22 day CCS (line 17); WT SOD1, 20-day-old WT SOD1 transgenic mice; WT SOD1/ CCS, 21- and 22-day-old WT SOD1/CCS (line 17) dual transgenic mice; G93A, 22-, 29- and 22-day-old G93A transgenic mice; G93A/CCS, 22-, 22- and 25-day-old G93A/CCS (line 17) dual transgenic mice.

Fig. S1B) there is no increased disulfide oxidation. We observed an averaged 4-fold increase in level of total disulfide-reduced SOD1 in mice analyzed by IAM (Fig. 2B), similar to what was seen in brain (Fig. 1B). Yet in the spinal cords of the mice analyzed, there is some increase in total SOD1 that parallels the increase in disulfide-reduced SOD1. Nevertheless, the end result is the same: CCS over-expression fails to increase oxidation of the G93A SOD1 disulfide in brain and spinal cord and if anything, disease in these animals correlates with increased levels of disulfide-reduced SOD1. This is completely the opposite of what is seen in non-diseased mice over-expressing WT SOD1 where CCS over-expression consistently promotes oxidation of the WT SOD1disulfide in both brain and spinal cord.

These effects of CCS over-expression appear specific to neuronal tissue. In the kidney, the WT or G93A SOD1 disulfide is virtually all disulfide-oxidized without CCS over-expression, and there are no increases in disulfide-reduced SOD1 in the kidney of G93A/CCS mice analyzed either by IAM (Fig. 3) or by AMS (Supplementary Material, Fig. S1B). However, as CCS expression is driven by the mouse prion protein promoter, the level of CCS over-expression in the kidney is modest compared with that of neuronal tissue (Fig. 3) (30).

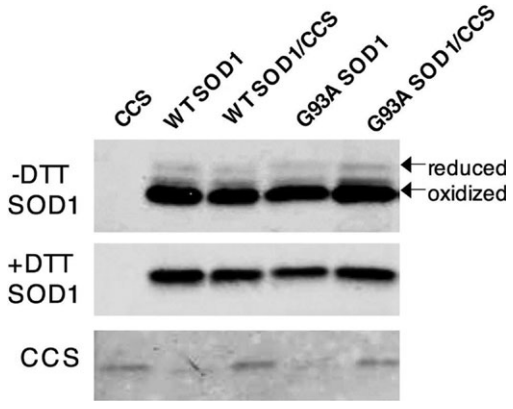

Figure 3. SOD1 disulfide analysis in kidney. The SOD1 disulfide was analyzed as in Figure 1A (with human-specific SOD1 antibody as in Figure 2A) using 3.0 μg kidney protein samples prepared in the presence of IAM from the individual mice indicated. Mice employed included: CCS, 29 day CCS (line 17) transgenic mouse; WT SOD1, 31-day-old WT SOD1 transgenic mouse; WT SOD1/CCS, 31-day-old WT SOD1/CCS (line 17) dual transgenic mouse; G93A, 29-day-old G93A transgenic mouse; G93A/CCS, 29-day-old G93A/CCS (line 17) dual transgenic mouse.

The effects of CCS over-expression in a cell culture model

To further investigate the effects of CCS on the disulfide of WT versus ALS-mutant SOD1 we used a cell culture model. In human HEK 293 or 293 FT cells, the disulfide of endogenous human SOD1 is largely oxidized (e.g. Fig. 4A, lane 11; Fig. 4B, lane 5), consistent with full activation of the enzyme by the CCS and/or CCS independent system in these cells. However, when SOD1 is over-expressed in these cells, the disulfide is largely reduced. This is the case with both WT SOD1 (Fig. 4A, lanes 2–4; Fig. 4B, lane 2) and G93A SOD1 (Fig. 4A, lanes 12–13) as well as ALS SOD1 mutants, A4V and G41D (Fig. 4B, lanes 6 and 9). The endogenous CCS-dependent and -independent pathways are not sufficient to fully activate over-expressed SOD1 in this cell culture model. When CCS was over-expressed in these cells, there was increased oxidation of the WT SOD1 disulfide (Fig. 4A, lanes 5–7; Fig. 4B, lane 3) as well as ALS mutants G93A (Fig. 4A lanes 14–15), A4V and G41D SOD1 (Fig. 4B, lanes 7 and 10). Yet full oxidation of the SOD1 disulfide was never achieved. In a side-by-side comparison, the WT SOD1 disulfide was oxidized roughly 50% (see numbers Fig. 4A, lanes 5–7) while that of the G93A SOD1 mutant was oxidized only $<$25% (lanes 14 and 15), even though CCS was over-expressed approximately 100-fold (see Supplementary Material, Fig. S3). The copper chaperone is not the only factor that limits activation of over-expressed SOD1, particularly in the case of the ALS SOD1 mutant (see Discussion).

In spite of this limited activity of over-expressed CCS, some visible increase in disulfide oxidation is apparent with G93A SOD1, which is certainly not the case with the G93A/CCS SOD1 mouse, suggesting that CCS is not fully active with the ALS mutant in neuronal tissues. To monitor effects of inactive CCS, we over-expressed in cell culture a CCS mutant in which both of the copper binding ligands of CCS domain III (C244,C246) were mutated to serine. Mutations in these residues disrupt the copper transfer and disulfide oxidation activities of the copper chaperone (32–34). C244,246S CCS was transiently co-transfected in HEK 293 or 293FT cells with either WT or various ALS-mutant SOD1s. As previously published (32), there was no increase in SOD1 activity with the inactive C224,246S CCS (data not shown). Unlike over-expressed WT CCS, expression of the C244S, C246S variant failed to enhance disulfide oxidation with either WT or ALS-mutant SOD1 (Fig. 4A, lanes 8–10, 16–17; Fig. 4B, lanes 4, 8 and 11) as expected. Surprisingly, in cases where the SOD1 disulfide was reasonably oxidized to begin with (e.g. WT SOD1 expressed in 293FT cells; Fig. 4A), there was a visible loss in disulfide oxidation (i.e. increase in disulfide reduction) with mutant CCS over-expression that is highly reproducible (note triplicate transfections of Fig. 4A). In cases where the basal level of oxidized disulfide was very low to begin with (e.g. G93A SOD1, Fig. 4A) loss of the disulfide was not easily discerned, however upon quantitation, a certain degree of disulfide loss was detected with all the SOD1s upon over-expression of the cys mutant CCS (see numbers Fig. 4). The C244S,246S variant still harbors all the CCS domain II sequences required for interacting with SOD1, and in studies with the yeast protein, the analogous C229, 231S mutant docks with SOD1, yet cannot activate the enzyme (35). Thus the interactions between C244S,246S CCS and SOD1 in this case are 'non-productive' at least at the level of the disulfide. Such interactions not only fail to promote disulfide oxidation, but can actually effect some accumulation of disulfide reduced SOD1. It is noteworthy that this same effect on the disulfide is observed in the neuronal tissues of G93A SOD1 mice over-expressing WT CCS (Fig. 1).

The effects of CCS over-expression on aggregation of ALS mutant SOD1 in cell culture

We investigated the effects of CCS over-expression on SOD1 misfolding as monitored by SOD1 insolubility. ALS mutant, but not WT SOD1 can show varying levels of insolubility in non-ionic detergents and this has been used widely as a marker for misfolding of mutant SOD1 in spinal cord of ALS-transgenic models and in culture models (13–20,22,23,36). We first examined the effects of CCS on SOD1 misfolding in cell culture. As seen in Figure 5A, a fraction of ALS-mutant (lanes 3, 5, 7, 9 and 11), but not WT SOD1 (lane 2) can be detected in the detergent-insoluble fraction ('SOD1-pellet') when expressed in HEK 293 cells in agreement with a previous report (17). Notably, co-transfection of CCS reduced the amount of detergent-insoluble SOD1 observed for each ALS-mutant tested (Fig. 5A, lanes 4, 6, 8, 10 and 12) and the same results were obtained in 293FT cells expressing G93A SOD1 (not shown). As such, it would appear that CCS over-expression protects mutant SOD1 from misfolding in cell culture. To confirm this, the opposite experiment was conducted using fibroblasts derived from $CCS^{-/-}$ versus $CCS^{+/+}$ mice also transgenic for G37R SOD1 (6,37). The absence of CCS correlated with increased levels of detergent-insoluble G37R SOD1 in the duplicate cell cultures analyzed (Fig. 5B). CCS can clearly help protect against SOD1 misfolding in this cell culture system.

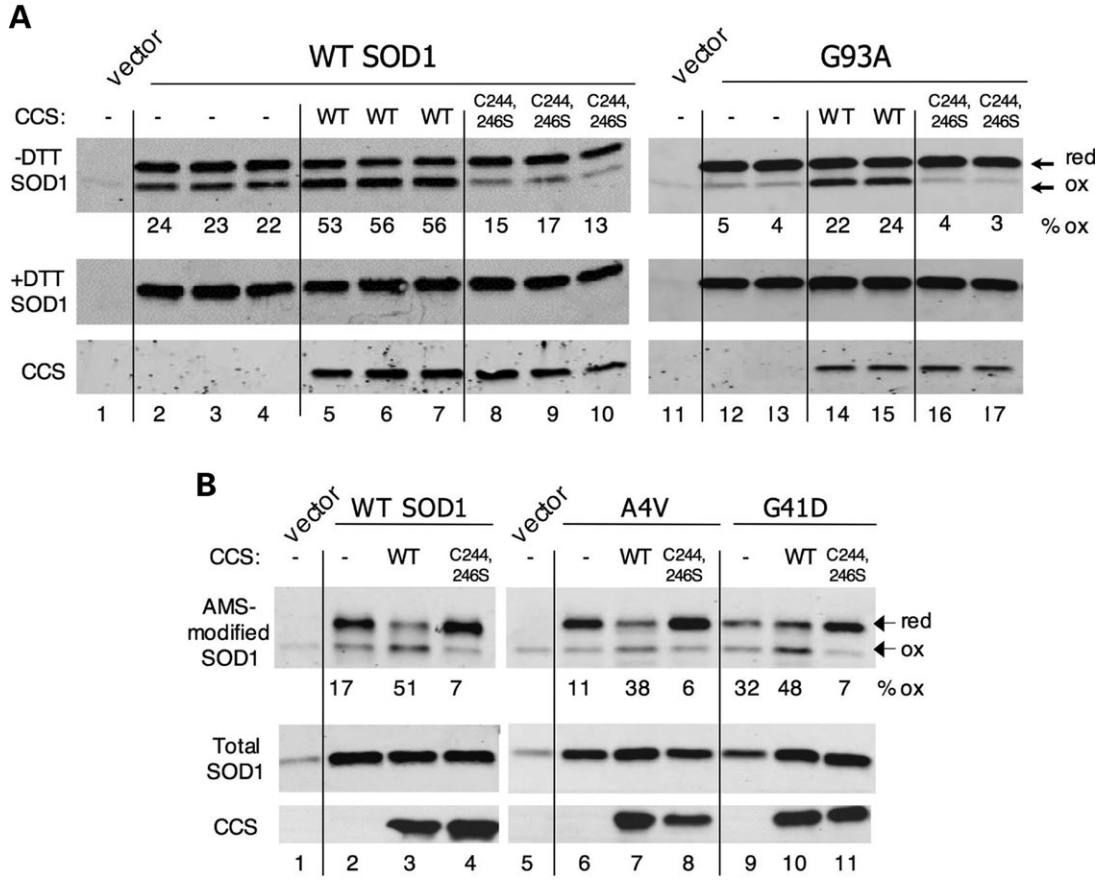

Figure 4. Effect of CCS over-expression on the SOD1 disulfide in cell culture. HEK 293FT (**A**) or HEK 293 (**B**) cells were transiently transfected with either WT or the various indicated ALS-mutant SOD1s in the pEF-BOS expression vector (17,43) or empty vector as indicated and co-transfected as needed with either WT or C244,246S mutant CCS under the CMV promoter. Cells were lysed in the presence of IAM (A), or in the presence or absence of AMS (B). (A) Protein from IAM treated lysates was subjected to non-reducing (-DTT) or reducing (+DTT) SDS–PAGE. Duplicates and triplicates represent independent transfections to demonstrate reproducibility. (B) AMS-treated and untreated fractions were subject to reducing SDS–PAGE. (A and B) Immunoblotting and quantitation of SOD1 proceeded as in Fig. 1A; numbers represent percent of disulfide-oxidized SOD1.

To determine if this apparent protective effect of CCS reflected its ability to promote oxidation of the mutant SOD1 disulfide, we employed the CCS domain III mutant C244,246S, which does not promote disulfide oxidation and can trend toward promoting some disulfide reduction (as in Fig. 4). Surprisingly, co-transfection of C244,246S CCS also reduced detergent-insoluble SOD1 (Fig. 6, lane 4). Hence CCS protection against SOD1 misfolding does not require disulfide oxidation nor copper loading. Physical interactions with SOD1 may be sufficient. To address this, we employed the CCS domain II mutant, Y134E,G135E. Domain II of human and yeast CCS physically interacts with SOD1 (35,38) through contacts that parallel SOD1-SOD1 homodimer interactions (3). Domain II residues Y134 and G135 of human CCS are predicted to stabilize the CCS-SOD1 heterodimer (3); indeed Y134E,G135E substituted human CCS cannot activate SOD1 (32) and the corresponding K136E, G137E yeast CCS cannot dock with SOD1 (35). As seen in Figure 6 (lane 5) over-expression of Y134E,G135E human CCS failed to reduce the level of misfolded A4V SOD1 as defined by detergent insolubility. Since C244,246S but not Y134,G135E CCS can protect against SOD1 misfolding, CCS interactions with SOD1 are sufficient to prevent SOD1 aggregation in this assay, even in the absence of disulfide oxidation. These studies implicate a role for CCS in proper folding of SOD1 that is separate from SOD1 enzyme activation.

CCS and SOD1 aggregation in ALS mutant mice

Since our cell culture studies demonstrated CCS protection against mutant SOD1 misfolding without disulfide oxidation (Fig. 6), it was curious that CCS over-expression did not ameliorate motor neuron disease in G93A SOD1 mice (30). We therefore investigated the effect of CCS over-expression on mutant SOD1 misfolding in the G93A/CCS mouse as defined by detergent solubility. As seen in Figure 7, insoluble mutant SOD1 is readily detected in spinal cord from two separate 10-month-old symptomatic G93A SOD1 mice and one 8-month-old symptomatic G37R spinal cord (Fig. 7, lanes 8–10); consistent with previous reports (17,18). As expected, detergent-insoluble SOD1 was not detected in the spinal cord from two very young asymptomatic G93A SOD1 mice (Fig. 7, lanes 4–5). Surprisingly, no detergent-insoluble G93A SOD1 was detected in spinal cord of symptomatic G93A/CCS mice of similar age

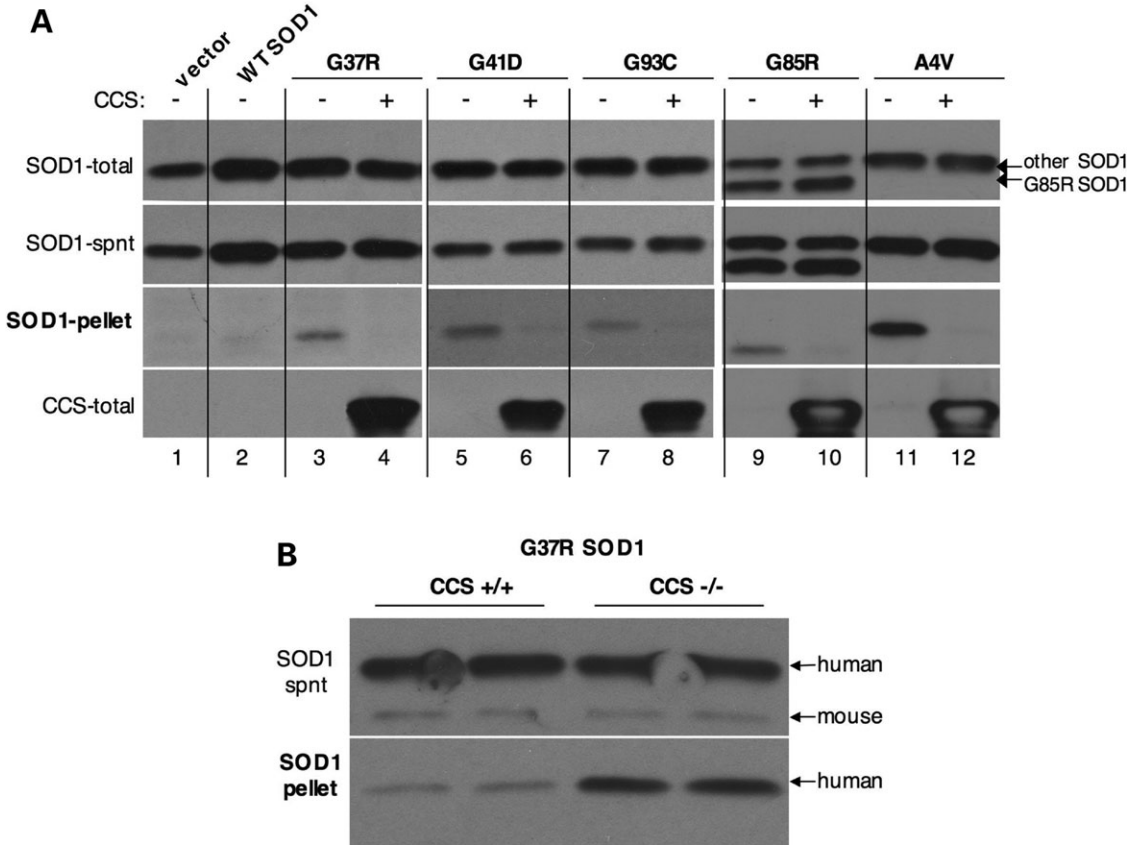

Figure 5. Effects of CCS on levels of detergent-insoluble SOD1 in cultured cells. Lysates prepared from the indicated cells were subjected to ultracentrifugation in the presence of 0.5% non-ionic detergent P-40 (NP-40) to obtain NP-40 soluble ('spnt') and NP-40 insoluble ('pellet') fractions. 50 μg of the unfractionated lysate ('total'), the NP-40-soluble ('spnt') and NP-40–insoluble ('pellet') fractions were subject to SDS–PAGE and immunoblotting with anti-SOD1 and, where indicated, re-probed with anti-CCS antibodies as described in Figure 1. A secondary horseradish-peroxidase-linked antibody was used and detection involved ECL. Cells used: (**A**) HEK 293 cells transiently co-transfected with vectors for expressing the indicated human SOD1s and for expressing CCS or co-transfected with empty vector to maintain constant levels of transfected DNA; (**B**) Fibroblast cell lines derived from CCS $^{+/+}$ and $^{-/-}$ G37R mice. Duplicates samples in (B) represent independent cultures.

(Fig. 7, lanes 6–7). To our knowledge, the diseased G93A/CCS mouse represents the first case of SOD1-induced motor neuron disease that is not associated with readily detectable levels of mutant SOD1 insolubility. Over-expressed CCS is accelerating disease in the mutant SOD1 mouse without formation of insoluble aggregates of SOD1.

DISCUSSION

Since it was first described as a copper chaperone for SOD1, CCS was predicted to have strong beneficial properties regarding the maturation, stability and enzymatic activity of SOD1. It was therefore quite surprising to note that CCS over-expression dramatically accelerated disease in a mouse model for SOD1-linked ALS (30), particularly since CCS should promote disulfide oxidation in SOD1 (5,6), and thereby protect against SOD1 misfolding and aggregation. Yet as demonstrated here, there is no such beneficial effect of CCS in the G93A/CCS transgenic mouse. CCS over-expression failed to enhance oxidation of the G93A SOD1 disulfide and if anything, was associated with some increase in disulfide-reduced G93A SOD1 in brain and spinal cord of the very young diseased mice (Fig. 1, 2).

Why would over-expressed CCS fail to enhance disulfide oxidation only with mutant SOD1 and only in tissues and not cell culture? The propensity of ALS SOD1 mutants, including G93A, to misfold particularly in the disulfide-reduced state (25,39) may make these SOD1s a less than optimal substrate for disulfide oxidation by CCS. Even in the cell culture system, over-expressed CCS has a reduced capacity to oxidize the disulfide of G93A SOD1 compared with that of WT SOD1 examined in parallel (Fig. 4A). CCS may still attempt to react with a pool of misfolded mutant SOD1, but cannot complete the activation process, leading to some increase in a pool of disulfide-reduced SOD1 (Fig. 8). We envision this pool of SOD1 to also lack the catalytic copper co-factor (Fig. 8), consistent with our analysis of G93A SOD1 enzyme activity (Supplementary Material, Fig. S2). Indeed, this same effect is observed in cell culture by over-expressing the C244,246S CCS mutant that has the capacity to dock SOD1, but cannot oxidize the disulfide nor transfer copper (Fig. 4).

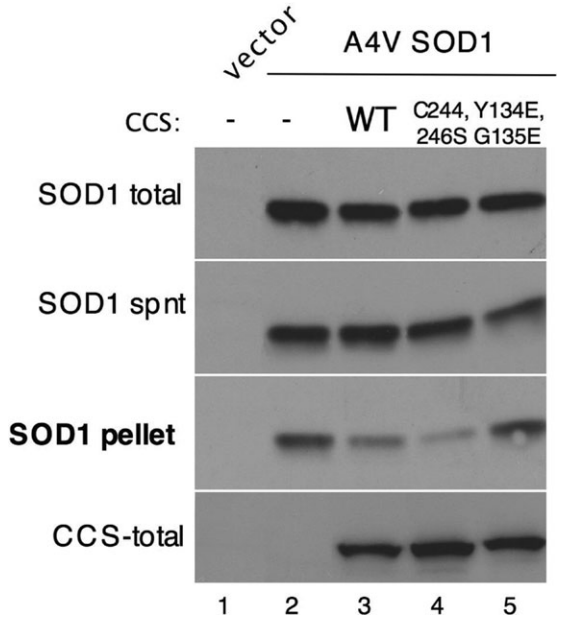

Figure 6. CCS protection against SOD1 insolubility does not require SOD1 activation. HEK 293FT cells were transiently transfected with A4V mutant SOD1 and co-transfected as needed with WT CCS or mutant variants C244,246S or Y134E,G135E prior to analysis of detergent insoluble SOD1 as described in Figure 5.

Perhaps the most universally detected biochemical alteration correlated with onset and progression of SOD1-induced motor neuron disease is the formation of detergent insoluble precipitates of SOD1 (13–20,22,23,36). However, we observed no such evidence of mutant SOD1 misfolding in the spinal cord of diseased G93A/CCS mice. Consistent with this, Son *et al*. (30). reported that ubiquitin-positive inclusions, which are normally a hallmark of late disease in G93A mice (12), are absent in symptomatic G93A/CCS mice, as are high-molecular-weight SOD1 species on denaturing gels (30). To our knowledge, this represents the first ALS mouse model where disease is not associated with the presence of detergent-insoluble SOD1. Even in the G93A SOD1 mouse where disease is slightly accelerated by over-expressing WT SOD1, there is mutant SOD1 aggregation (13). If such aggregation is a component of disease in the CCS/G93A mouse, it must involve a fraction of SOD1 that is so minute it escapes detection by conventional methods. More likely, SOD1 aggregation is not necessary for disease in this case.

If SOD1 aggregation is not involved, then what is causing disease in the CCS/G93A mice? Son *et al*. (30). reported that these mice display marked mitochondrial pathology in the spinal cord and exhibit a higher level of mutant SOD1 associated with mitochondrial fractions from the spinal cord (30). SOD1-mediated damage to the mitochondria may be the cause of disease. SOD1 must be in the disulfide-reduced state in order for it to enter the mitochondria (40,41) and the increased level of disulfide-reduced SOD1 in G93A/CCS mice could account for the increased mitochondrial SOD1 (Fig. 8). In addition to mitochondrial pathology, another model for mutant SOD1-induced disease involves the extracellular toxicity of secreted mutant SOD1; it has been postulated that the disulfide-reduced form of SOD1 is recruited to the secretory pathway in this case (42) (Fig. 8).

Lastly, these studies on CCS over-expression have provided new insight into the biology of this copper chaperone. CCS is best known for its role in inserting copper and oxidizing the SOD1 disulfide (2,5,6,10), yet our studies support a role for CCS independent of SOD1 enzyme activation. In a cell culture model, over-expressed CCS can help prevent against mutant SOD1 misfolding as monitored by detergent insoluble SOD1 (Fig. 5). Analysis with CCS mutants indicates that physical interactions with SOD1 are required, but not copper insertion nor disulfide oxidation (Figs 4 and 6). As such, CCS may serve as molecular chaperone for SOD1 independent of its role as a copper chaperone. CCS can clearly affect the SOD1 polypeptide in the absence of enzyme activation, yet as revealed in the G93A/CCS mouse, such effects of CCS can be detrimental.

MATERIALS AND METHODS

Tissues, plasmids and cell lines

Plasmids encoding human WT, G41D, G37R, A4V, G93C and G85R SOD1 cDNAs in the pEF-BOS vector backbone have been described elsewhere (17,43) and were a kind gift from Dr David Borchelt. The G93A SOD1 mutant was created by site-directed mutagenesis of WT SOD1 in the pEF-BOS vector (QuickChange kit, Stratagene). The human CCS expression vector (pJP001) was created by mobilizing human CCS (sequences −3 to +286 from the yeast expression plasmid pSMCCS) (2) by *Xba*I and *Bam*H1 restriction digestion, and by ligating at the *Nhe*1 and *Bam*HI restriction sites of pcDNA3.1+ (Invitrogen). As such, human CCS falls under control of the CMV promoter. The C244,246S and Y134E,G135E CCS mutants were derived from pJP001 through site-directed mutagenesis (QuickChange kit, Stratagene). The DNA sequence integrity of all plasmids was verified by double-stranded DNA sequencing (The Johns Hopkins University Core facility). Immortalized G37R SOD1/CCS$^{+/+}$ and G37R SOD1/CCS$^{-/-}$ fibroblasts derived from abdominal skin of adult mice have been previously described (6,37). HEK 293FT cells were obtained from Invitrogen. Tissue samples from perfused CCS over-expressing and control mice were snap frozen prior to analysis. Frozen G37R SOD1 (line 29) spinal cord was a gift from Dr David Borchelt.

Cell culture and transfection conditions

CCS$^{+/+}$ and $^{-/-}$ fibroblasts were maintained as previously described (6). HEK 293 and HEK 293FT cells were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with either 10% fetal bovine serum/1 mM L-glutamine or 10% fetal bovine serum/2 mM L-glutamine/1 mM L-pyruvate/1:100 MEM non-essential amino acids (Invitrogen)/500 ug/ml Geneticin respectively, using standard culture procedures. Transient transfections in HEK 293 and HEK 293FT were performed on >90% confluent cells in 60 mm dishes (poly-d-lysine coated for FT cells; BD Biocoat) using Lipofectamine 2000 transfection reagent

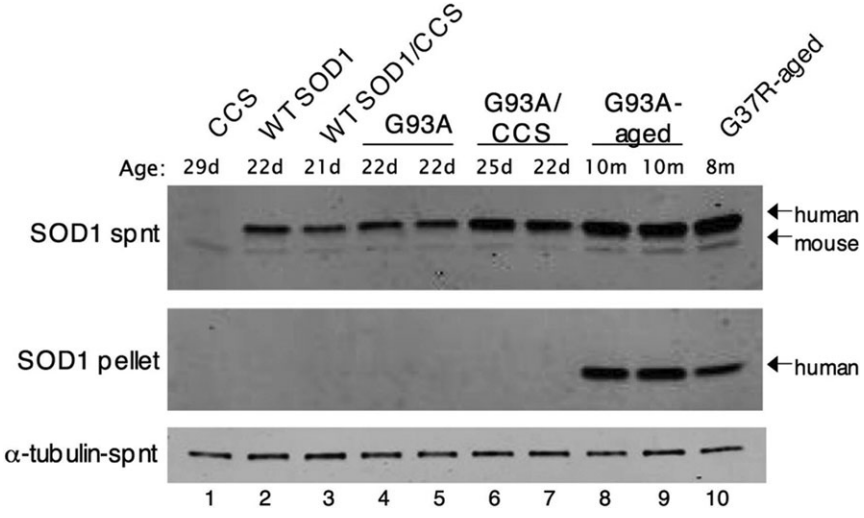

Figure 7. Absence of insoluble SOD1 in spinal cord of G93A/CCS mice. Spinal cord lysates from the designated mice at the noted ages were prepared in 0.5% NP-40 subjected to ultracentrifugation as in Figure 5. Two micrograms total protein for each fraction (spnt, pellet) was subject to SDS–PAGE and immunoblotting using the anti-bodies and the LI-COR Odyssey imager detection system described in Figure 1. Mice used in lanes 1, 2, 3, 4, 5, 6, 7 correspond to mice used in lanes 1, 2, 4, 6, 8, 11, 10 of Figure 2A, respectively. G93A/CCS, G93A-aged, and G37R (line 29)-aged mice were symptomatic at the ages sampled.

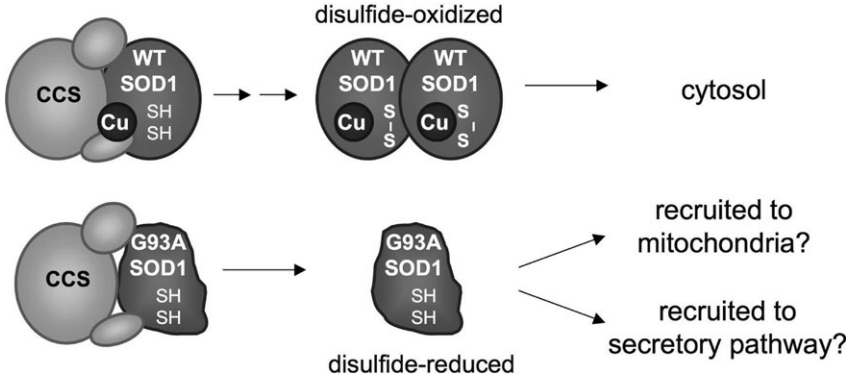

Figure 8. Possible fates of disulfide reduced SOD1 in the G93A/CCS mouse. Shown are possible models for how reduction of the SOD1 disulfide may be on the pathway to disease in the G93A/CCS mouse. While over-expressed CCS interacts productively with WT SOD1 to oxidize the disulfide, a fraction of misfolded G93A SOD1 is a poor substrate for over-expressed CCS and remains in the disulfide-reduced state, resulting in SOD1 recruitment to either the mitochondria or the secretory pathway.

(Invitrogen) (10 μl/500 μl for a 60 mm plate). For experiments involving co-transfection of SOD1 and CCS expression plasmids, the total amount of transfected DNA was kept constant between different conditions using empty pcDNA3.1+ for SOD1-only conditions. Cells were harvested 24 h later for biochemical analyses.

Biochemical assays

SOD1 disulfide analysis by IAM was performed as described (29,31) with slight modification. Specifically, tissue and cells were homogenized by sonication in buffer containing 50 mM Hepes/2.5% SDS/0.1 mM EDTA (pH 7.2) with 100 mM IAM (1:20 w:v or 400μl/60 mm dish respectively) and lysates were incubated at 37°C for 30 minutes. Total protein concentration was determined by Bradford assay. Samples were prepared in either the presence or absence of 400 mM DTT and boiled in SDS sample buffer prior to polyacrylamide gel electrophoresis on pre-cast 14% Tris–glycine gels (Invitrogen). Prior to transfer onto nitrocellulose membranes the proteins were subjected to in-gel reduction by shaking for 10 min in transfer buffer containing 2% β-ME to increase antibody recognition of disulfide-oxidized SOD1 as described (29). The alternative AMS-modification assay for disulfide analysis was performed as previously described (4) for cultured cells, as well as for tissues with slight modification. Extra steps were taken for tissues to minimize artificial oxidation during homogenization. Frozen tissue samples (in 1:10 w/v lysis buffer ± 15 mM AMS) (4) were ground to a powder using a mortar and pestle cooled with liquid nitrogen. A second equal volume of lysis buffer ± 15 mM AMS was added after thawing and samples were vortexed before incubation for 1 h at 37°C. This method for tissue processing was found to not effect oxidation of a purified reduced A4V SOD1 spiked into brain tissue (not shown). The assay for detergent-insoluble SOD1 in tissue and cells was performed

as described (17). Lysates for SOD1 activity analysis were prepared as previously described by freeze-thaw in phosphate-buffered saline (6). SOD1 activity was assayed by non-denaturing gel electrophoresis at 4°C and staining with nitro blue tetrazolium (44,45), and a second denaturing gel was run for SOD1 immunoblotting.

Antibodies, western blotting and quantitation

Human-specific and mouse/human SOD1 anti-sera were generated against peptides comprising amino acids 24–36 and 124–136 of human SOD1, respectively, and were used at 1:1000 dilution. Affinity-purified anti-sera generated against a hexahistidine-tagged fusion protein corresponding to residues 31–274 of mouse CCS, which reacts well with human CCS, obtained from Dr Jeffrey Rothstein, was used at 1:500 dilution. Monoclonal anti alpha-tubulin antibody (Sigma T6199) was used at 1:2000 dilution. For experiments involving ECL detection, horse-radish peroxidase-conjugated secondary-antibody incubations were performed at 1:10 000 dilution and blots were subject to densitometric tracing using NIH ImageJ software. For experiments involving near-infrared fluorescence scanning detection using the LI-COR Odyssey imager, incubation with 1:5000 dilution of either Alexa Fluor goat anti-rabbit 680 or goat anti-mouse 680 secondary antibody (Invitrogen) was used and quantitation was performed using LI-COR Odyssey software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. none declared.

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