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CuATSM Protects Against the *In Vitro* Cytotoxicity of Wild-Type-Like Copper−Zinc Superoxide Dismutase Mutants but not Mutants That Disrupt Metal Binding

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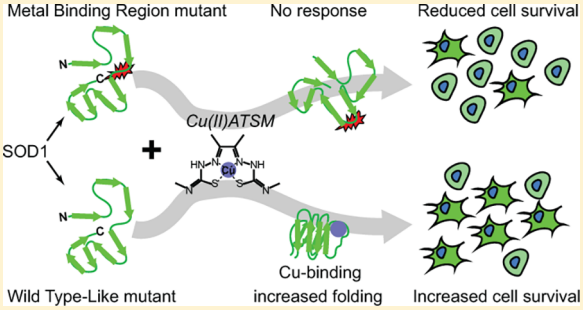
Supporting Information

ABSTRACT: Mutations in the *SOD1* gene are associated with some forms of familial amyotrophic lateral sclerosis (fALS). There are more than 150 different mutations in the *SOD1* gene that have various effects on the copper−zinc superoxide dismutase (SOD1) enzyme structure, including the loss of metal binding and a decrease in dimer affinity. The copper-based therapeutic CuATSM has been proven to be effective at rescuing neuronal cells from SOD1 mutant toxicity and has also increased the life expectancy of mice expressing the human transgenes $SOD1^{G93A}$ and $SOD1^{G37R}$. Furthermore, CuATSM is currently the subject of a phase I/II clinical trial in Australia as a treatment for ALS. To determine if CuATSM protects against a broad variety of SOD1 mutations, we used a well-established cell culture model of SOD1-fALS. NSC-34 cells expressing SOD1-EGFP constructs were treated with CuATSM and examined by time-lapse microscopy. Our results show a concentration-dependent protection of cells expressing mutant $SOD1^{A4V}$ over the experimental time period. We tested the efficacy of CuATSM on 10 SOD1-fALS mutants and found that while protection was observed in cells expressing pathogenic wild-type-like mutants, cells expressing a truncation mutant or metal binding region mutants were not. We also show that CuATSM rescue is associated with an increase in human SOD1 activity and a decrease in the level of SOD1 aggregation *in vitro*. In conclusion, CuATSM has shown to be a promising therapeutic for SOD1-associated ALS; however, our *in vitro* results suggest that the protection afforded varies depending on the SOD1 variant, including negligible protection to mutants with deficient copper binding.



KEYWORDS: *CuATSM, copper, protein aggregation, SOD1, ALS*

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease and is the most common type of motor neuron disease. ALS causes loss of muscle control and eventually leads to death due to respiratory failure, generally within 3−5 years of diagnosis.[1] These symptoms manifest due to the degeneration of upper and lower motor neurons in the motor cortex and spinal cord. The lifetime risk for ALS is ∼1:300 to 1:400,[2] and currently, there are no effective treatments. Most cases of ALS are sporadic (sALS); however, approximately 10% are inherited. These familial cases (fALS) are linked to mostly missense mutations in more than a dozen genes, including *SOD1*,[3] *TARDBP*,[4] *FUS*,[5,6] and *CCNF*.[7] Furthermore, the most common genetic cause of ALS results from hexanucleotide repeats in noncoding regions of the *C9ORF72* gene.[8,9]

SOD1 was the first gene identified to contain mutations associated with fALS[3] and is the most widely studied of the currently known ALS-associated genes. More than 150 (mostly missense) mutations occurring throughout the protein sequence are currently thought to be fALS causative, with these mutations being responsible for ∼20% of fALS cases.[10] The *SOD1* gene encodes an antioxidant enzyme, copper−zinc superoxide dismutase (SOD1), which functions as a cytosolic free radical scavenger. The protein has a homodimeric

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structure where monomers contain an intramolecular disulfide bond and also bind one copper (Cu) atom and one zinc (Zn) atom.[11] The maturation pathway of SOD1 is thought to proceed through binding of Zn to the nascent polypeptide,[12] followed by association with the copper chaperone for SOD1 (CCS), which delivers Cu and facilitates disulfide formation.[13] Cu is essential for its superoxide scavenging role as it reacts with oxygen free radicals in the following Cu-dependent reactions:[14]

$$O_2^{\bullet-} + Cu^{II}ZnSOD \rightarrow O_2 + Cu^{I}ZnSOD \quad (1)$$

$$O_2^{\bullet-} + 2H^+ + Cu^{I}ZnSOD \rightarrow H_2O_2 + Cu^{II}ZnSOD \quad (2)$$

It was initially thought that loss of enzymatic function was responsible for disease; however, this was determined not to be the case as many SOD1-fALS mutants retain enzymatic activity at near wild-type levels.[15] SOD1-fALS mutations are broadly designated into two classes: (i) wild-type-like (WTL) mutations, which have dismutase activity levels similar to that of the wild-type protein, and (ii) metal binding region (MBR) mutations, which occur within the metal binding region of SOD1 that disrupt proper coordination of either Zn or Cu, resulting in decreased folding stability and enzymatic activity.[11] The existence of the WTL mutants suggests that pathology is caused by a toxic gain of function, most likely due to the misfolding and subsequent aggregation of mutant SOD1.[11]

A growing body of evidence suggests that there is an association between protein aggregate formation and the development of ALS.[16] In SOD1-fALS, it has been shown that affected neurons often have proteinaceous inclusions primarily composed of SOD1, and the aggregation propensities of SOD1 mutants correlate with cell death *in vitro*.[17] Also, the prion-like propagation of SOD1 misfolding and aggregation has been proposed to explain the progressive nature of the disease.[18−21] Aggregation of the SOD1 enzyme is generally associated with mutations that result in the destabilization of the structure because of a decreased level of metal binding,[22] defective disulfide bond formation,[23] an increased level of dimer dissociation,[24] and a reduction in monomer folding stability.[25] In addition, SOD1 is significantly metastable in motor neurons primarily because of its extremely high level of expression,[26] making it prone to aggregation, and it is expected that mutations act to exacerbate this situation.

Recently, the Cu-based small molecule Cu(II)ATSM (CuATSM) has been used experimentally to rescue ALS phenotypes in cell and mouse models.[27−29] Furthermore, human trials are currently underway as a potential treatment for ALS.[26] In separate experiments, CuATSM was shown to increase the level of survival of mice overexpressing the human SOD1 transgene with a G37R[28,29] or G93A[27,30] mutation, each of which is a WTL mutant. CuATSM was effective in protecting against SOD1-fALS-mediated pathology in these experiments; however, because both mutations tested were WTL,[11] whether CuATSM is still effective against MBR mutants is unknown. Therefore, using an established cell model of SOD1-related fALS,[17,31,32] we set out to investigate whether CuATSM is protective across a range of ALS-associated SOD1 mutations. In this work, 10 SOD1-fALS mutants ($SOD1^{A4V}$, $SOD1^{G127X}$, $SOD1^{C6G}$, $SOD1^{G37R}$, $SOD1^{H46R}$, $SOD1^{E100G}$, $SOD1^{D90A}$, $SOD1^{G93A}$, $SOD1^{V148G}$, and $SOD1^{G85R}$) and wild-type SOD1 ($SOD1^{WT}$) were used to examine mutant-specific effects of CuATSM treatment (Figure 1). We found that of these mutants, cells expressing $SOD1^{H46R}$, $SOD1^{G127X}$, and $SOD1^{G85R}$, all which have disrupted Cu binding, were not protected by CuATSM. In addition, we found that CuATSM significantly reduces the level of aggregation of pathogenic WTL mutants. These results are consistent with CuATSM delivering Cu to mutant SOD1 when Cu binding is not impaired, increasing protein stability, reducing the level of aggregation, and improving cell survival.

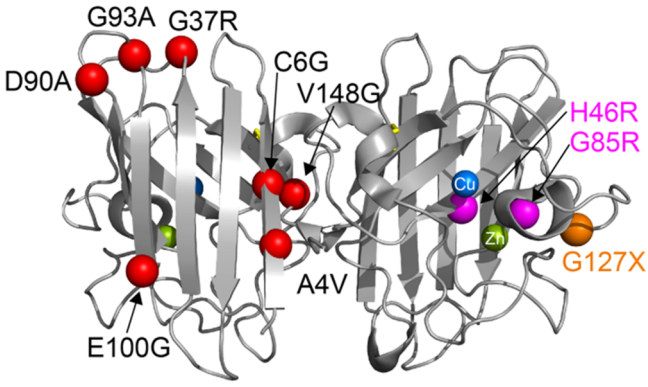

Figure 1. SOD1 dimer with post-translational modifications and ALS-associated mutants. The left subunit shows the location of the wild-type-like (WTL) SOD1 mutants examined in this study (red spheres). The right subunit shows the Cu (blue sphere) and Zn (green sphere) ions in their native binding sites. The metal binding region (MBR) mutants H46R and G85R (magenta) are shown close to the metal binding pocket. The truncation mutant G127X is shown as an orange sphere. The disulfide bond is shown on both subunits (yellow sticks). Protein Data Bank entry 1HL5.[33]

RESULTS AND DISCUSSION

The therapeutic benefits of CuATSM have been experimentally proven in multiple models of SOD1-fALS[27−30] and independently verified in the SOD1-G93A mouse model;[34] however, the exact mechanism of action in these models remains to be elucidated. Previous *in vivo* studies have used CuATSM on SOD1-fALS mice carrying transgenes for the WTL mutants $SOD1^{G37R}$[29] and $SOD1^{G93A}$;[30] however, no experiments have been performed in models expressing SOD1 MBR mutants. Here, we have addressed this gap in knowledge by using CuATSM to treat a cell model of SOD1-fALS that allowed for rapid comparison of a broad range of mutants, finding that CuATSM alleviated pathology only for pathogenic WTL SOD1 mutants.

CuATSM Protects NSC-34 Cells against Mutant SOD1 Toxicity in a Dose-Dependent Manner. CuATSM has been shown to improve the survival and locomotor function of SOD1 mice in a dose-dependent manner.[29] Here we sought to determine the effect of CuATSM on the survival of a well-established cell model, NSC-34 cells overexpressing human mutant SOD1-EGFP.[17,31] Using live cell time-lapse microscopy, we monitored the survival of NSC-34 cells transiently transfected with $SOD1^{WT}$-EGFP (Figure 2A), $SOD1^{A4V}$-EGFP (Figure 2B), or the EGFP only control (Figure 2C) over a 90 h period in the presence of increasing concentrations (0−1 μM) of CuATSM. Consistent with what has been observed in transgenic SOD1 animal models,[29] CuATSM increased the rate of survival of mutant SOD1-expressing NSC-34 cells in a dose-dependent manner. In the absence of CuATSM, $SOD1^{A4V}$-EGFP expression resulted in a significant loss of

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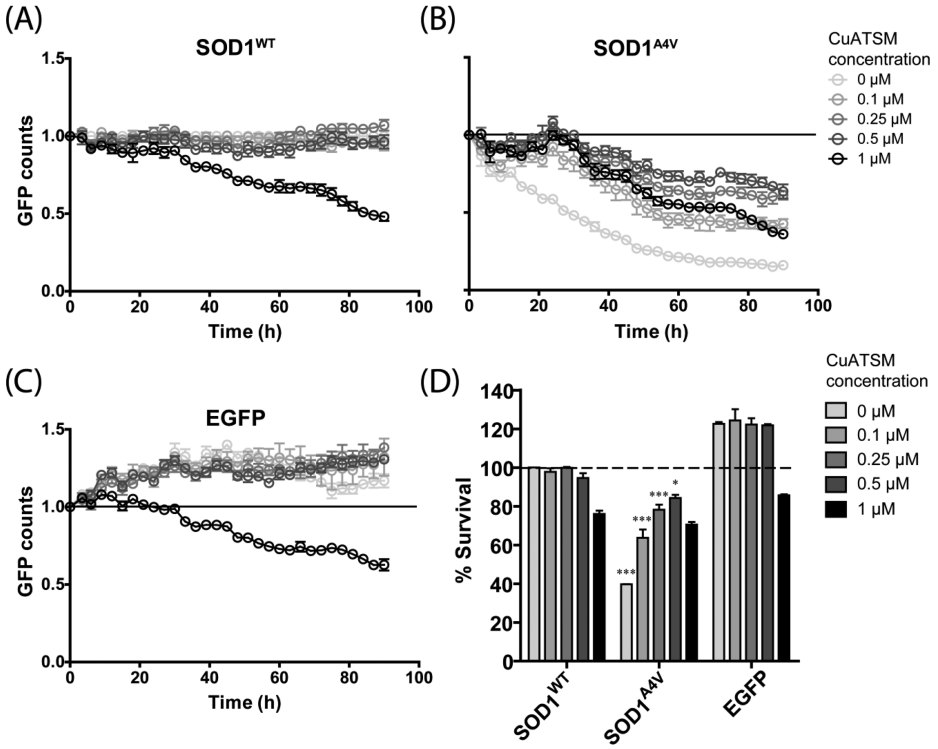

Figure 2. Effects of CuATSM on the survival of NSC-34 cells expressing SOD1-EGFP. NSC-34 cells expressing (A) $SOD1^{WT}$, (B) $SOD1^{A4V}$, and (C) EGFP were treated with 0−1 μM CuATSM, and images were acquired for 90 h every 3 h on an IncuCyte automated fluorescent microscope in the GFP channel. The number of GFP positive cells was determined at every time point and first normalized to the starting time point after which the value at each time point for mutants was normalized to the number of untreated $SOD1^{WT}$ cells at corresponding time points (indicated by the solid straight line in panels B and C). (D) Percentage survival of cells expressing $SOD1^{WT}$, $SOD1^{A4V}$, or EGFP in the presence of 0−1 μM CuATSM determined by measuring the area under the GFP count curves and normalizing to $SOD1^{WT}$ in the absence of CuATSM. Data shown are means ± the standard error of the mean (SEM) ($n = 3$). Each sample is an individually treated single microplate well. Two-way analysis of variance with the Bonferroni post-test was used to compare differences. Asterisks indicate a significant difference from $SOD1^{WT}$ (*$p < 0.05$; ***$p < 0.001$).

cells compared to those expressing $SOD1^{WT}$-EGFP. A concentration-dependent increase in $SOD1^{A4V}$-EGFP cell numbers was observed post-CuATSM treatment, except for that at the 1 μM dose, which resulted in a loss of cells relative to the 0.5 μM dose (Figure 2D). The protection afforded to $SOD1^{A4V}$-EGFP-expressing cells by CuATSM was maximal at 0.5 μM. Treatment with 1 μM CuATSM was also toxic to cells expressing both $SOD1^{WT}$-EGFP and EGFP. Therefore, a CuATSM concentration of 0.5 μM was selected as the most appropriate dose at which to examine the toxicity associated with overexpression of mutant SOD1 in NSC-34 cells for subsequent experiments.

CuATSM Does Not Protect Cells Expressing SOD1 Mutations That Disrupt Metal Binding. Although CuATSM has been previously shown to rescue SOD1 mouse models from overexpression of either $SOD1^{G37R}$[28,29] or $SOD1^{G93A}$,[27−30] it has not been tested against other SOD1 mutations, some of which are MBR mutations that affect Cu coordination. Having determined an effective dose of CuATSM to apply to our model, we transiently transfected NSC-34 cells with 10 different mutant SOD1-EGFP constructs to determine the effect of CuATSM on a range of SOD1 mutants, including MBR mutations.

As we have shown previously, expression of human SOD1 mutants results in a loss of cell numbers relative to cells expressing $SOD1^{WT}$.[17] An exception to this is the D90A mutation that is recessive in some Scandinavian families.[35] In fact, the pathogenic nature of D90A is contested, with some suggesting heterozygosity is enough to cause disease,[36] while others have found that as many as 2.5% of the Northern Swedish population are carriers, suggesting D90A is not pathogenic.[37] Consistent with this is the fact that some D90A kindred individuals without the mutation inherit ALS.[38] Our results are consistent with the latter in that expression of $SOD1^{D90A}$ did not produce a phenotype and as such essentially acts as an additional WT control. In the case of cells expressing EGFP fused to $SOD1^{A4V}$, $SOD1^{C6G}$, $SOD1^{G37R}$, $SOD1^{E100G}$, $SOD1^{G93A}$, and $SOD1^{V148G}$, the addition of 0.5 μM CuATSM resulted in an increased number of SOD1-EGFP positive cells compared to the number of untreated cells at all time points (Figure 3A). After calculation of the area under the curve across the entire time course, it is apparent that CuATSM treatment significantly improved transfected cell survival for these mutants (Figure 3B).

Cells transfected with MBR mutants $SOD1^{H46R}$ and $SOD1^{G85R}$ also exhibited a loss of transfected cells relative to that of $SOD1^{WT}$ over time. Similarly, the truncation mutant $SOD1^{G127X}$, which fails to fold and thus should not bind metals, also resulted in relative cell loss. However, addition of CuATSM did not alter the rate of cell loss (Figure 3A), and no significant differences in cell survival were observed with the addition of CuATSM in these mutants (Figure 3B). Cells expressing $SOD1^{D90A}$, EGFP, or $SOD1^{WT}$ were also unaffected by CuATSM, but this was most likely due to the fact that expression of these constructs did not result in a significant loss of cells when they were left untreated.

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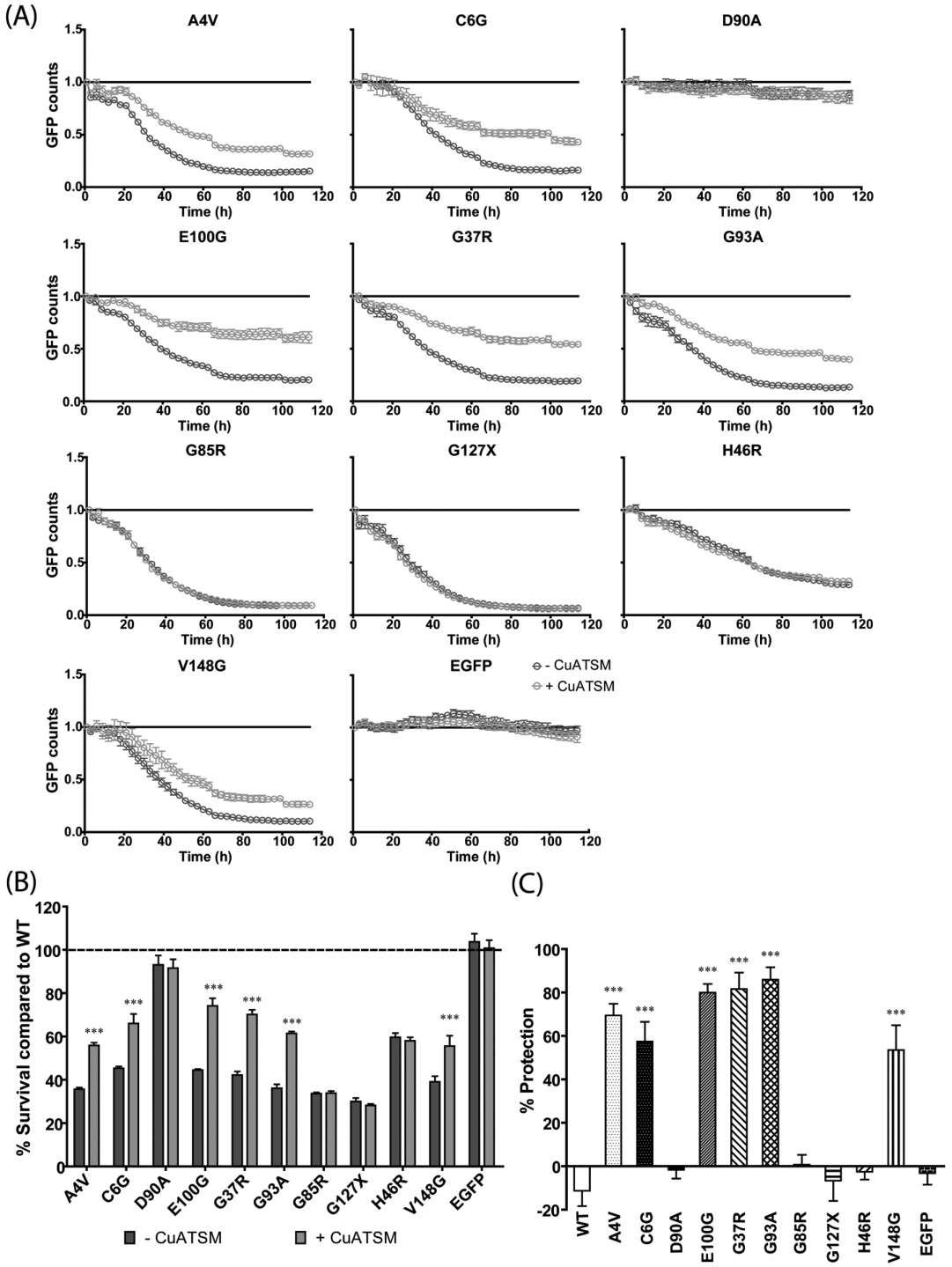

Figure 3. Effect of CuATSM on the toxicity of different SOD1 variants. (A) NSC-34 cells were transfected with SOD1-EGFP constructs, and GFP fluorescence was monitored over 120 h in the presence and absence of 0.5 μM CuATSM. (B) Cell survival compared to cells expressing $SOD1^{WT}$ was determined by measuring the area under the curve for each SOD1 variant in the presence and absence of CuATSM over the 120 h time period. Data shown are means ± the standard error of the mean (n = 3). Each sample is an individually treated, single microplate well. Data are representative of three independent experiments. Statistical significance was determined using two-way analysis of variance with the Bonferroni post-test (***p < 0.001). (C) The protection provided to cells by CuATSM was determined by calculating the difference between each variant with and without CuATSM treatment as a percentage relative to untreated cells. Data shown are means ± the standard deviation (n = 3). Each sample is an individually treated, single microplate well. Data are representative of three independent experiments. Statistical significance was determined using one-way analysis of variance with a Tukey's multiple comparison post-test. Asterisks indicate significant differences with respect to $SOD1^{WT}$ (***p < 0.001).

Each pathogenic SOD1 mutant elicited a variable effect on relative NSC-34 cell survival (Supplementary Figure 1). To compare the amount of protection afforded to each mutant by CuATSM, a protection score (% protection) was calculated. The score represents the percentage protection CuATSM provides to cells compared to the vehicle control, regardless of

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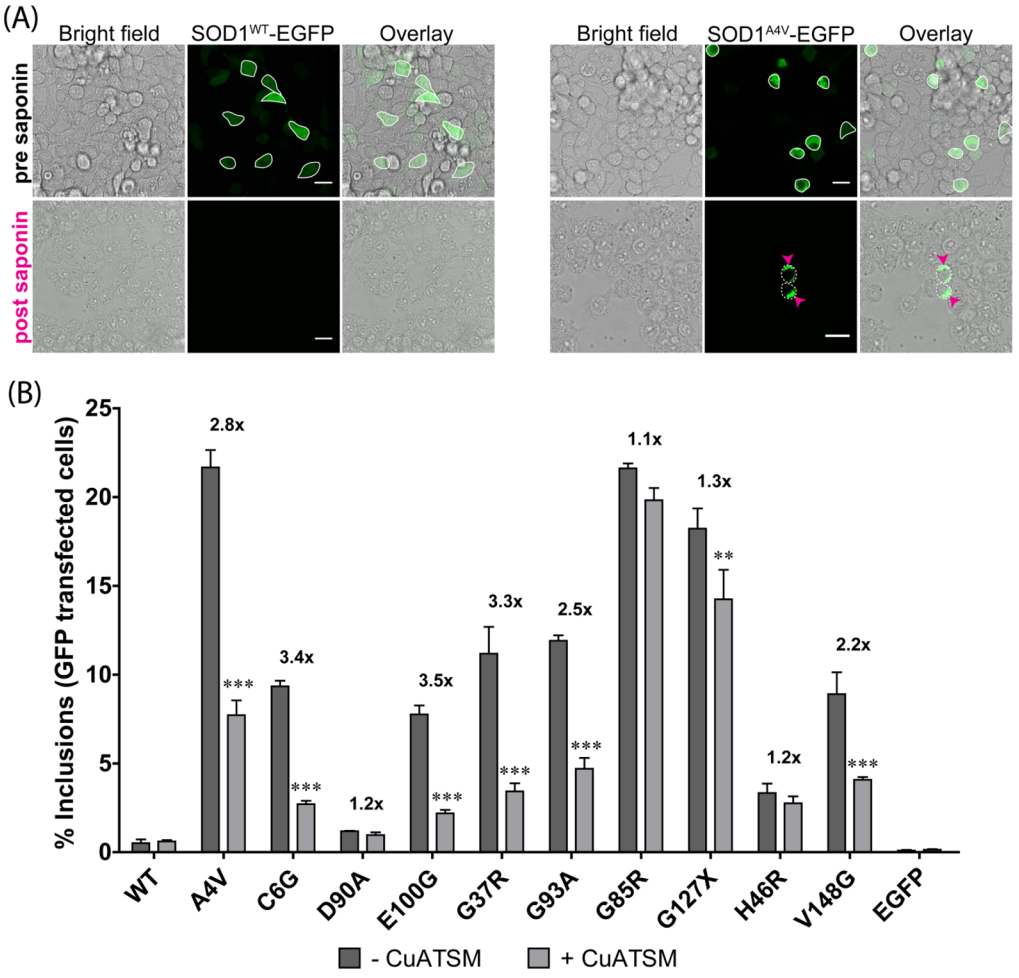

Figure 4. CuATSM decreases the level of aggregation of WTL SOD1 mutants. (A) NSC-34 cells transfected with $SOD1^{WT}$-EGFP or $SOD1^{A4V}$-EGFP were imaged by confocal microscopy pre- and post-saponin treatment to identify cells containing insoluble SOD1-EGFP aggregates. Scale bars are 20 μm, and pink arrowheads highlight insoluble aggregates. (B) NSC-34 cells expressing SOD1-EGFP constructs were also imaged pre- and post-saponin treatment using an IncuCyte automated microscope to determine the proportion of transfected cells that contained SOD1-EGFP inclusions. Data shown are means ± the standard error of the mean (n = 3). Each sample is an individually treated, single microplate well. Data are representative of three independent experiments. The fold decrease in the number of cells containing aggregates seen with CuATSM treatment (relative to the vehicle control, −CuATSM) is stated above each mutant. Statistical significance was determined by two-way analysis of variance with the Bonferroni post-test (**p < 0.01; ***p < 0.001).

differences in cell loss from $SOD1^{WT}$. This allows for direct comparisons across SOD1 variants. The analysis confirmed that cells expressing $SOD1^{G127X}$, $SOD1^{H46R}$, and $SOD1^{G85R}$ were not protected by CuATSM (Figure 3C). Cells expressing $SOD1^{WT}$, $SOD1^{D90A}$, or EGFP were also not protected by CuATSM treatment. For all other SOD1 mutants, CuATSM had a significant protective effect on cell survival. Protection scores for these WTL mutants ranged from ∼50% ($SOD1^{V148G}$) to ∼85% ($SOD1^{G93A}$), suggesting CuATSM does not provide protection to all WTL mutants equally (Figure 3C and Supplementary Table 1). However, there was no relationship between protection score and associated clinical disease severity (Supplementary Figure 2).

The results reported here show that addition of CuATSM to cells expressing MBR SOD1-fALS mutants and truncation mutant $SOD1^{G127X}$ does not alleviate the toxicity of these mutant proteins. Furthermore, CuATSM does not affect cells expressing EGFP, $SOD1^{D90A}$, or $SOD1^{WT}$. However, as seen in previously published data from mouse models,[27−30] we show that CuATSM improves the survival of cells expressing pathogenic WTL SOD1 mutants.

CuATSM Decreases the Level of Aggregation of SOD1 Mutants in Cultured Cells. Although SOD1 is a small protein, it requires extensive processing and post-translational modification (PTM) to reach maturity,[13] which includes Zn binding, Cu binding, and disulfide formation. These PTMs impart remarkable stability to the enzyme, making it resistant to proteolytic digestion,[39] thermal unfolding,[40] and chemical denaturation.[41] Removal of these PTMs is considered necessary for its misfolding and aggregation.[42] Indeed, the thermal stability of mature mutant $SOD1^{G93A}$ is highly similar to that of $SOD1^{WT}$,[43] with significant differences between the stability of mutants becoming apparent only in the apo state. Considering this, the availability of additional Cu imparted by CuATSM treatment would be expected to aid CCS in its chaperone function[44] and increase the level of maturation of the pool of intracellular SOD1 to a Cu-bound form that is less susceptible to misfolding and aggregation.

To test whether CuATSM affects the aggregation of SOD1 in the NSC-34 cell model, cells expressing the various SOD1-EGFP constructs were treated with 0.5 μM CuATSM for 72 h before cell membranes were permeabilized with saponin[45,46] to

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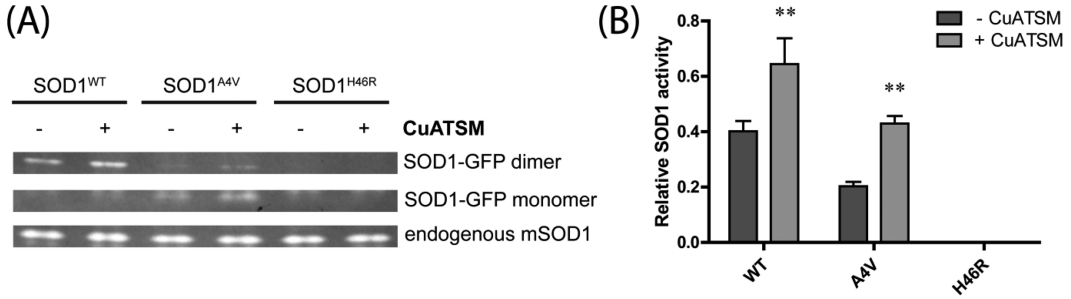

Figure 5. CuATSM increases the activity of WT-like SOD1 mutants. (A) Lysates from NSC-34 cells overexpressing $SOD1^{WT}$, $SOD1^{A4V}$, and $SOD1^{H46R}$ were separated on native 4 to 16% gradient bis-tris gels, and SOD1 activity was determined by in-gel zymography. Equal loading was determined by measuring endogenous mouse SOD1 on the same gel. (B). Quantification of SOD1 activity expressed relative to endogenous mouse SOD1 levels. Data shown are means ± the standard error of the mean (n = 3). Data are from three independent experiments. Two-way analysis of variance with the Bonferroni post-test was used to compare differences between cell lysates cultured in the presence and absence of CuATSM (**p < 0.01).

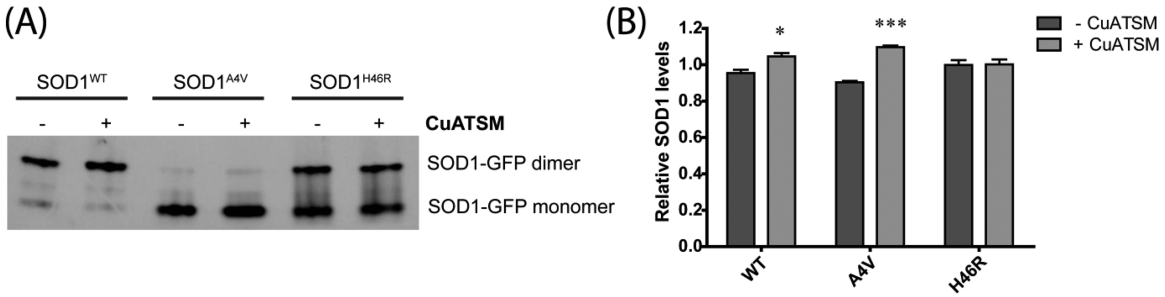

Figure 6. CuATSM increases the levels of WT-like SOD1 mutants. (A) Cell lysates from NSC-34 cells overexpressing $SOD1^{WT}$, $SOD1^{A4V}$, and $SOD1^{H46R}$ were separated on native 4 to 16% gradient bis-tris gels, and SOD1-EGFP levels were determined by Western blot analysis. (B) SOD1-EGFP levels were quantified from Western blot analyses by densitometry and expressed relative to the average of the total EGFP signal for that variant. Data shown are means ± the standard error of the mean (n = 3). Data are from three independent experiments. Two-way analysis of variance with the Bonferroni post-test was used to compare differences between cell lysates cultured in the presence and absence of CuATSM (*p < 0.05; ***p < 0.001).

allow the diffusion of soluble protein out of the cell and facilitate examination of the insoluble EGFP signal remaining inside the cell[47] (Figure 4A). The amount of insoluble material (aggregates) remaining inside cells was then quantified as a proportion of transfected cells. We found that aggregation varied among SOD1 variants with as many as 22% of $SOD1^{A4V}$-expressing cells and ∼1% of $SOD1^{WT}$- and $SOD1^{D90A}$-expressing cells containing aggregates (Figure 4B). In cells in which minimal aggregation occurred, specifically EGFP, $SOD1^{WT}$, and $SOD1^{D90A}$, CuATSM had no significant effect on aggregation. We also found that CuATSM did not significantly reduce the level of aggregation of MBR SOD1 mutants $SOD1^{H46R}$ and $SOD1^{G85R}$ [1.2- and 1.1-fold reductions, respectively, in the percentage of cells containing aggregates compared to the vehicle control (Figure 4B)]. Cells expressing largely unstructured $SOD1^{G127X}$ also underwent a small but significant decrease in levels of aggregation in the presence of CuATSM (∼1.3-fold reduction). In contrast, cells expressing pathogenic WTL mutants all showed a significant reduction in cells containing aggregates [on average ∼3-fold reduction (also see Supplementary Table 1)], distinguishing them from the MBR mutants and controls (Figure 4B).

Although Cu deficiency is associated with an increased level of misfolding of SOD1, it has previously been shown that treatment with CuATSM did not reduce the levels of misfolded SOD1 in $SOD1^{G37R}$ mice.[28] Interestingly, CuATSM has been shown to reduce the level of accumulation of abnormally phosphorylated and fragmented TDP-43 in the spinal cords of mice expressing low levels of mutant $SOD1^{G93A}$.[48] This may be due to direct effects on SOD1 aggregation as SOD1 aggregates have been shown to induce TDP-43 mislocalization.[49] Here, we show that CuATSM treatment reduces the level of aggregation of pathogenic WTL SOD1 mutants and has small but nonsignificant effects on the aggregation of MBR mutants and a small but significant effect on cells expressing $SOD1^{G127X}$. Similarly, CuATSM does not alter the minimal aggregation in cells expressing $SOD1^{D90A}$, EGFP, or $SOD1^{WT}$. Our data suggest that the primary effect CuATSM has on aggregation is to supply Cu to mutant SOD1, thereby stabilizing its structure. The small reductions in the level of aggregation observed with mutants unable to bind Cu suggest that CuATSM could also have additional indirect effects on aggregation by activating stress pathways via Nrf2,[50] including the ubiquitin-proteasome system, but that this modest effect does not extend to cell viability. In the case of G127X, there is also a chance that because it retains the Cu-coordinating histidine residues (H46, H48, H63, and H120), Cu could be binding with a low affinity. This could afford a slight increase in stability that plausibly translates to the minimal effect on aggregation observed here.

CuATSM Increases SOD1 Levels and Activity in WT-like Mutants. Previous work demonstrates that administration of CuATSM results in an increase in human mutant SOD1 protein levels in mouse models of ALS.[27,28] The increase in SOD1 protein levels also corresponded to an increase in SOD1 activity,[27] suggesting the additional protein

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was properly folded and metalated. To test whether SOD1 levels and activity were increased by CuATSM in the NSC-34 cell model used here, Western blotting and in-gel zymography were performed on cell lysates from cells cultured in the absence or presence of CuATSM. Cells expressing $SOD1^{WT}$, $SOD1^{A4V}$, and $SOD1^{H46R}$ were compared as examples of WTL and MBR mutants. In-gel zymography revealed that CuATSM increased the activity of human SOD1 in the case of cells expressing $SOD1^{WT}$ and $SOD1^{A4V}$ (Figure 5 and Supplementary Figure 3). Similarly, CuATSM increased the activity of $SOD1^{D90A}$ and all pathogenic WTL mutants tested (Supplementary Figure 3). However, $SOD1^{A4V}$ remained predominantly monomeric regardless of treatment. This increase in activity was accompanied by increases in protein levels as determined by Western blot analysis (Figure 6 and Supplementary Figure 4). In contrast, $SOD1^{H46R}$ was inactive in the presence and absence of CuATSM (Figure 5B), and there was no difference in protein levels after treatment with CuATSM (Figure 6B). In-gel zymography results also indicate that the MBR mutant $SOD1^{G85R}$ and truncation mutant $SOD1^{G127X}$ remain inactive regardless of CuATSM treatment (Supplementary Figure 3).

Large pools of Cu deficient SOD1 have been detected in the spinal cords of $SOD1^{G37R}$ mice, and treatment with CuATSM reduced the size of this pool and subsequently increased the amount of fully metalated SOD1.[28] Here we show that CuATSM treatment increases the concentration of the active enzyme and reduces the level of aggregation of SOD1 mutants in NSC-34 cells. These results suggest that, in our system, overexpression of pathogenic WTL SOD1 mutants exceeds the basal Cu demands of the cell, creating a pool of Cu deficient SOD1 that misfolds and aggregates. Interestingly, overexpression of $SOD1^{WT}$ and $SOD1^{D90A}$ also results in a pool of Cu deficient SOD1 as treatment with CuATSM increases human SOD1 activity in NSC-34 cells; however, this does not result in aggregation or toxicity. This is consistent with the notion that a misfolded conformer is required for toxicity and not Cu deficiency per se. While our results suggest that misfolding and aggregation are the common attributes of WTL and MBR mutants that are associated with toxicity, Cu deficiency could result in the misfolding of SOD1 and subsequent aggregation and toxicity. Indeed, as aggregated SOD1 propagates in a prion-like fashion,[18−21] increasing the availability of Cu to cells could reduce the size of the pool of misfolded SOD1 required for template-driven aggregation and thus propagation.

Copper homeostasis is an area of growing interest in SOD1-fALS research as Cu is found to be dysregulated in animal models expressing either WTL or MBR SOD1 mutants.[51−54] For example, Cu levels are found to be increased in affected neural tissues in SOD1-fALS mouse models, including $SOD1^{G37R}$, $SOD1^{G93A}$, and $SOD1^{WT}$, and even in mice expressing the MBR double mutant $SOD1^{H46R/H48Q}$.[51−54] This suggests that Cu dysregulation in the central nervous system (CNS) is a common feature of SOD1 overexpression. The SOD1-associated Cu dysregulation in these models has been shown to affect other Cu binding proteins such as cytochrome *c* oxidase[30] and ceruloplasmin.[55] Although this effect appears to be partially model-specific as cytochrome *c* oxidase activity was no different from controls in the latter study, this difference is likely due to the fact that the hCCS × $SOD1^{G93A}$ model is an extremely severe model of the disease. Furthermore, Cu dysregulation appears to occur early in SOD1 mouse models where changes to Cu levels in the CNS are detected as early as 30 days of age, well before the onset of symptoms, in $SOD1^{G93A}$ mice.[56] Similarly, the $SOD1^{G37R}$ mouse model experiences a redistribution of Cu from the liver to the CNS at the embryonic stages of development, which is not seen in adult mice,[57] suggesting changes to Cu regulation over time in SOD1 mouse models.

Considering the work described above, there have been several proposals for the molecular mechanism(s) by which CuATSM attenuates the ALS phenotype in model systems. These proposals emphasize the role of CuATSM in restoring healthy SOD1 folding and function, or they emphasize the role of CuATSM in restoring Cu homeostasis to other proteins as well as SOD1. The interpretation is made difficult because of the concomitant effects of SOD1 misfolding and Cu dysregulation.

Evidence of a homeostatic role includes research in which treating a $SOD1^{G93A}$ mouse model with CuATSM was shown to inhibit peroxynitrite activity.[48] Levels of oxidative and nitrosative stress were also found to be reduced, although it is unclear if this resulted from the increased level of folding of SOD1. Also, the transgenic mouse line overexpressing human CCS and $SOD1^{G93A}$ (hCCS × $SOD1^{G93A}$) was found to have severe Cu dyshomeostasis, where treatment with CuATSM restored Cu binding to cytochrome *c* oxidase.[30] This model also presented the curious observation that levels of misfolded SOD1 did not decrease even though levels of Cu-bound SOD1 increased.[27] Other effects of CuATSM not related to the CNS have been reported, such as upregulation of antioxidant enzymes in human coronary artery smooth muscle cells and cardiac myocytes through activation of transcription factor NF-E2 related factor 2 (Nrf2).[50] Finally, several Parkinson's disease mouse models[58] were shown to have better outcomes upon treatment with CuATSM, consistent with the idea that misfolded nonmetalated SOD1 is not required for its protective ability in neurodegeneration.

Our results, however, indicate distinct differences among MBR mutants, a truncation mutation, and pathogenic WTL mutants regarding the effect of CuATSM on the toxicity, aggregation, and maturation of SOD1 mutants in NSC-34 cells (Figure 7). This evidence supports a distinct role of CuATSM in alleviating SOD1 aggregation and toxicity for only mutants that are capable of being rescued. However, cultured cells do not effectively model the CNS, and it is likely that CuATSM has multiple modes of action in the complex environment of the brain, including on cell types other than neurons (e.g., astrocytes[59]). Future work examining the effect of CuATSM in SOD1 MBR mutant transgenic mouse lines will be needed to reconcile these data. Furthermore, the role of metals in neurodegeneration is still unclear. Most data linking aberrant metal interactions to neurodegeneration in humans come from ecological and occupational studies.[60] It is possible that metal homeostasis, like proteostasis, declines or changes with age. More research into this important area is necessary to determine the exact links between neurodegeneration and metal homeostasis.

CONCLUSIONS

Our results suggest that CuATSM is protective against toxicity mediated by a range of pathogenic WTL SOD1 mutants but not in the case of MBR mutants or a truncation mutant in a cell culture system. These results may be relevant for the design of future clinical trials of CuATSM for MND patients,

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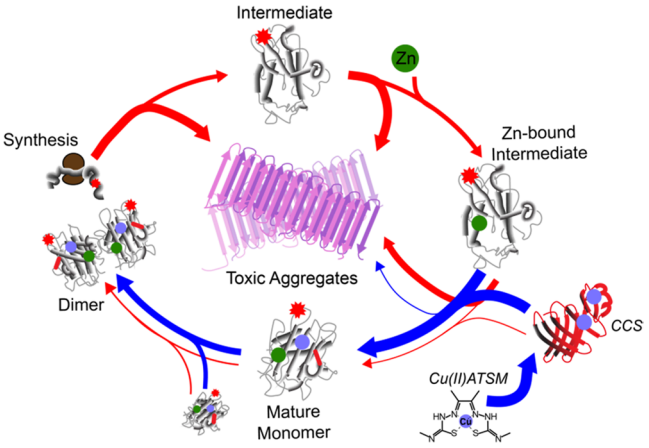

Figure 7. Model of CuATSM rescue of mutant SOD1 pathology. Red arrows show the folding and off-folding pathways for SOD1, whereas blue arrows show the contribution of CuATSM to these pathways. The arrow thickness suggests the probability of the pathways occurring. Following synthesis, mutant SOD1 (red star) folds into an intermediate state that is primed for Zn binding. Zn-bound SOD1 associates with Cu-loaded CCS for transfer of Cu, leading to the formation of a mature SOD1 monomer that can form dimers. CuATSM results in a larger pool of Cu-bound CCS, which in turn results in greater transfer of Cu to SOD1, reducing the amount of SOD1 that enters an off-folding pathway at this point.

as the disease type (fALS or sALS) and specific SOD1 mutations linked to the fALS case may both have an impact on the efficacy of the treatment.

METHODS

Plasmids. The pEGFP-N1 vectors containing human SOD1 sequences were generated as described previously.[61]

Cell Culture and Transfection. Neuroblastoma × spinal cord hybrid NSC-34 cells[62] were maintained in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12) supplemented with 10% (v/v) fetal bovine serum (FBS, Bovogen Biologicals, Australia). Cells were maintained at 37 °C in a humidified incubator with 5% (v/v) atmospheric CO_2. Cells were transiently transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions with 0.5 μg of DNA per well for a 24-well plate and 2.5 μg of DNA per well for six-well plates.

CuATSM Treatment. Cu(II)ATSM (SYNthesis med chem, Parkville, Australia) was dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock. NSC-34 cells were treated with 0−1 μM CuATSM or DMSO vehicle control (diluted in culture medium) 24 h post-transfection for ≤120 h.

Time-Lapse Imaging. The viability of cells expressing SOD1 in the presence and absence of CuATSM was monitored over 120 h in an IncuCyte automated fluorescent microscope (Essen BioScience) as described in ref 17. NSC-34 cells were plated in 24-well plates at a confluency of ∼65% and transfected with SOD1-EGFP constructs 24 h later. Cells were dissociated 24 h post-transfection and replated in clear flat-bottom 96-well plates at a confluency of 30% in phenol red free DMEM/F12 containing 10% FBS and 0−1 μM CuATSM or vehicle control (DMSO). Images were acquired every 3 h and analyzed using a processing definition created to select GFP positive cells. The number of GFP positive cells was normalized to time zero before mutant SOD1 numbers were adjusted to $SOD1^{WT}$ values as previously reported.[17] To analyze differences between treatments, area under the curve (AUC) analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). The percent protection score was calculated from GFP count data normalized to time zero, where the AUC from 0 to 120 h was calculated for both vehicle control and CuATSM-treated cells. The following equation was used:

$$\%\ \text{protection} = 100 \times \frac{\text{AUC CuATSM} - \text{AUC vehicle}}{\text{AUC vehicle}}$$

Saponin Treatment To Detect Insoluble Aggregates. NSC-34 cells expressing SOD1-EGFP were gently treated 72 h post-transfection with 0.03% saponin in PBS to allow the diffusion of soluble intracellular SOD1-EGFP out of the cytoplasm to identify cells containing SOD1 aggregates.[45,46] Cells were imaged pre- and post-saponin treatment on a TCS SP5 confocal microscope (Leica, Germany) or by using the scan on demand function on the IncuCyte, and the number of cells containing aggregates was determined as a proportion of GFP-expressing cells.

Cell Lysis. NSC-34 cells grown in six-well plates and transfected with SOD1-EGFP were harvested 48 h post-CuATSM treatment (72 h post-transfection) with trypsin/EDTA (Gibco). Cells were washed with PBS before being resuspended in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% TX-100, 0.1% SDS, 10 mM NEM, 1 mM sodium orthovanadate, and Halt Protease Inhibitor Cocktail (Thermo Scientific)]. The protein concentration was determined by a BCA assay.

SOD1 In-Gel Zymography. SOD1 enzymatic activity was determined using in-gel zymography methods described previously.[63] Briefly, 30 μg of cell lysate or 100 ng of purified $SOD1^{WT}$ protein (prepared as previously described[17]) was loaded onto a NativePAGE 4 to 16% gradient bis-tris gel (Invitrogen). Following electrophoresis (150 V, 3 h), gels were incubated in 5 mM nitrotetrazolium chloride for 15 min with gentle rocking protected from the light. Gels were then rinsed with water before incubation with 30 μM riboflavin and 10 mM tetramethylethylenediamine (TEMED) for 20 min with gentle rocking in the dark. Gels were subsequently developed by exposure to fluorescent light on a light box until a significant signal was detected (achromatic bands). Gels were imaged on the Amersham Imager 6600RGB, and the band intensity was quantified using ImageJ version 1.48.[64] SOD1-EGFP activity was subsequently determined after normalizing the signal to purified SOD1 and endogenous mouse SOD1 levels.

Western Blotting. Following in-gel zymography, proteins were transferred onto a nitrocellulose membrane (Pall Corp.) using native transfer buffer [48 mM Tris and 39 mM glycine (pH 9.2) containing 0.04% (w/v) SDS]. The membrane was then incubated in native transfer buffer containing 20% methanol before the GFP signal was detected by fluorescence (excitation at 460 nm) on the Amersham Imager 6600RGB. Equal loading of the gel was determined by staining membranes with Ponceau red.

Statistical Analyses. Normally distributed data sets were analyzed for statistical significance via the following tests: one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test when comparing the means of multiple data sets and two-way ANOVA with The Bonferroni post-test when assessing multiple variables.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.8b00527.

Supplementary Table 1, Supplementary Figures 1−4, and references (PDF)

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M.R.Y. performed experiments, analyzed data, and wrote the initial manuscript. L.M. designed experiments, analyzed data, and edited the manuscript. S.S.P. provided feedback on experimental results and edited the manuscript. N.E.F. designed experiments, performed experiments, analyzed data, and wrote and edited the manuscript. J.J.Y. conceived of the study, designed experiments, analyzed data, and edited the manuscript.

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Notes

The authors declare no competing financial interest.

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