Scholarly Article Template

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Pellentesque tincidunt lobortis orci non venenatis. Cras in justo luctus, pulvinar augue id, suscipit diam. Morbi aliquet fringilla nibh, vel pellentesque dui venenatis eget. Orci varius natoque penatibus et magnis dis parturient montes, nascetur ridiculus mus. Donec ultricies ultrices magna gravida porta. Maecenas accumsan diam dui, auctor ornare ex pellentesque id. Integer tempus massa id augue finibus convallis. Nulla vestibulum, nisl id tempor pulvinar, felis dui pellentesque lacus, quis bibendum metus enim sed ex.

Introduction

Approximately 20% of familial human Amyotrophic Lateral Sclerosis (fALS) cases are attributed to mutations in the gene encoding Cu, Zn Superoxide Dismutase (SOD) (Rosen et al., 1993). Mice highly over-expressing mutant human SOD recapitulate the human disease closely, presenting with upper and lower motor neuron loss, progressive paralysis, and death in ˜150 days (Gurney et al., 1994).

[we still don't know how SOD causes disease, but here are some hypotheses. immature SOD is significantly destabilized and aggregation prone. some evidence suggests immature SOD in a soluble form may have toxic effects, too]

SOD normally undergoes a series of posttranslational modifications that activate and stabilize the protein: forming an intramolecular disulfide bond, binding one copper atom and one zinc atom per monomer, and dimerizing. However, a large fraction of SOD accumulates as zinc-containing, copper-deficient protein in the spinal cords of mice overexpressing mutant SODG37R or SODG93A[JM1] (Roberts et al., 2014; Williams et al., 2016). The human Copper Chaperone for SOD (CCS) catalyzes SOD copper binding and formation of the SOD intramolecular disulfide via a process requiring oxygen (Banci et al., 2012; Brown et al., 2004; Furukawa et al., 2004; Lamb et al., 2000). Co-expression of human CCS with human SOD in mice instigates a systemic copper deficiency early in development that is linked to rapid development of cellular and behavioral deficits. Mice co-expressing CCS[JM2] with "low" levels of SODG93A exhibit diminished copper-dependent cytochrome *c* oxidase (COX) activity compared to nontransgenic mice, rapidly develop [movement impairment] within 2 weeks and die within 20-50 days (Son et al., 2007; Williams et al., 2016). Mice expressing "high" levels of SODG93A with CCS die even sooner, within 3 weeks (Williams et al., 2016).

Treatment with the copper delivery agent CuATSM (diacetyl-bis(N4-methylthiosemicarbazone) rescues this early developmental crisis, restores COX activity, restores additional copper to SOD, and massively extends survival of high-expressing SODG93AxCCS mice from ˜15 days to over 600 days, which is significantly longer than the SODG93A lifespan of ˜150 days (Williams et al., 2016). CuATSM treatment also extends survival of SODG93A or SODG37R mice not co-expressing CCS (Hilton et al., 2017; McAllum et al., 2013; Roberts et al., 2014; Williams et al., 2016). Chemical reduction of the ligated CuATSM copper (II) atom is proposed to trigger copper release *in vivo*, resulting in Cu delivery to the CNS (Donnelly et al., 2012).

We hypothesized that chemical derivatives of CuATSM that are measurably easier to reduce will deliver more copper to the CNS and more robustly improve the SOD and COX measures of copper deficiency seen in SODxCCS mice. Here we test this reductive release model by administering CuATSM derivatives to mice co-expressing SODWT with CCS, a model which we show experiences lethal non-ALS developmental copper deficiency without intervention. [this is important because. . .]

Summary of Results

Here we report that human SODWT, when co-expressed with human CCS in mice, causes lethal copper deficiency that is rescue-able by treatment with CuATSM, but not by more-easily-reducible CuATSM chemical derivatives. [This suggests that there is more to the mechanism of CuATSM than getting lots of copper to the CNS.]

SODWTxCCS mice experience an early development crisis similar to G93AxCCS. They exhibit motor deficits, diminished CNS Cu-dependent COX activity, accumulated CNS Cu-deficient SOD, and death within 3 weeks. We designed a number of CuATSM derivatives based on the reductive release hypothesis. All of the derivatives delivered copper to the CNS, as measured by increased COX and Cu-replete SOD, but not all treatments rescued the early development crisis.

[JM1]what about in human patients?

[JM2]should talk about how CNS is only over-expressed in CNS, how Cu-deficiency is probably only in CNS

Results

SODWTxCCS mice die in $<$21 days, are rescued by CuATSM We previously showed that mice co-expressing CCS with a large number of copies[JM1] of SODG93A experienced an early development crisis that is rescuable by treatment with CuATSM. Here we report that mice co-expressing CCS with SODWT[JM2] also experience an early development crisis that is rescuable by treatment with CuATSM. SODWTxCCS pups were identifiable as runts compared to CCS littermates within 6 days after birth. Locomotive deficits such as dragging rear legs and falling over began to manifest immediately around the time littermates started to walk, or about 10 days after birth. By 15 days, most SODWTxCCS pups were immobile and unable to right themselves. SODWTxCCS mice died at an average of ??? days without intervention (Figure 1). SODWTxCCS mice have previously been reported to be symptom-free (Son et al., 2007), yet here we report that they exhibit symptoms similar to those of SODG93AxCCS ani-

Reserved for Publication Footnotes

mals (described by Son et al., 2007; Williams et al., 2016). Our CCS-expressing mice (without human SOD) are indistinguishable from nontransgenic animals, as previously reported.

Continuous treatment with CuATSM rescued the early development crisis seen in SODWTxCCS mice and extended survival beyond 400[JM3] days (Figure 1). Interestingly, treatment from 6 days until weaning at 21 days was also sufficient to rescue the early development crisis in SODWTxCCS mice, and animals in this group continued to live to ???[JM4] days without further treatment.

[JM5]

Figure1 - SODWTxCCS mice die early, are rescued by CuATSM. Caption pending figure replacement.

COX is deficient, likely cause for early death It has been proposed that the early death seen in SODG93AxCCS mice is a result of cytochrome *c* oxidase (COX) deficiency caused by redistribution of copper, via CCS, to SOD instead of COX (Williams et al., 2016). We assayed COX activity in CNS tissue from SODWTxCCS mice to investigate the link with the early development crisis.

SODWTxCCS mice exhibited diminished COX activity compared to CCS littermates, which were in turn similar to nontransgenic animals (Figure 2). Treating SODWTxCCS mice with CuATSM restored some COX activity, but levels remained below those of CCS or nontransgenic mice. SODWT-overexpressing mice also exhibited diminished COX activity compared to CCS or nontransgenic mice, though less severe[JM6] . Contrary to previous reports, these results suggest that overexpression of SODWT, especially with CCS, is capable of disturbing copper homeostasis, which manifests as COX deficiency.

[JM7] [JM8]

Figure2 – CuATSM restores COX activity in SODWTxCCS mice. Mice expressing SODWT (

)have decreased COX activity compared to CCS-only littermates (

) or nontransgenic mice (

). This difference is measurable six[JM9] days after birth. Co-expression of CCS with SODWT causes severely decreased COX activity (

) compared to nontransgenic mice. This COX deficiency in SODWTxCCS mice fails to be corrected without intervention and likely leads to these animals' death around 15 days[JM10] . Treatment with CuATSM partially restores COX activity levels (

), after an initial delay period suggesting Cu uptake in the CNS is limiting.

SODWT is metal deficient in the brains of SODWTxCCS miceSOD accumulates as copper deficient in the CNS of mice over-expressing mutant human SOD (Roberts et al., 2014; Williams et al., 2016). To investigate the link between SOD maturity and the early development crisis seen in SODWTxCCS mice, we assayed the maturity of human SODWT in CNS tissue of SODWTxCCS mice. Using native protein mass spectrometry methods previously described by (Rhoads et al., 2011, 2013), we measured the tissue concentrations of six SODWT subspecies differentiated by metal state (apo; 1-metal; Cu,Zn) and C57-C146 disulfide state (SH or S-S). Because immature wild-type human SOD is more stable than comparably immature mutant SOD (Furukawa and O'Halloran, 2005), we reasoned that SODWT would accumulate more readily in immature forms than would SODG93A. In practice though, the final difference between SODWT and SODG93A CNS tissue is not substantial[JM11] , probably because the SODWT gene copy number is much lower than in SODG93A mice.

SODWTxCCS mice at 15 days expressed about 100uM transgenic SODWT in brain. About half the SODWT was Cu, Zn, S-S SODWT, while the other half was mostly 1-metal and disulfide-reduced (SH) (**Figure 3**). Treating SODWTxCCS mice with CuATSM significantly decreased the fraction of SODWT that was copper-deficient, as well as the fraction that was SH, while not significantly changing the total concentration of SOD.

[JM12]

Figure3 - CuATSM restores copper to SODWT. Representative mass spectrum generated from brain homogenate of 15-day-old SODWTxCCS mice. Spectra were deconvoluted and quantitated by comparing human SODWT with the internal standard bovine SODWT. SODWTxCCS mice express about 100uM SODWT in the brain at 15 days. Half this pool of SODWT is "mature", containing two metals and the C57-C146 disulfide. Treatment with CuATSM significantly increases the fraction of SOD containing two metals and the C57-C146 disulfide.

In summary, treatment with CuATSM rescued the early development crisis seen in SODWTxCCS mice (**Figure 1**) and restored copper to brain COX (**Figure 2**) and SOD (**Figure 3**). Next, using COX and SOD as biomarkers for copper delivery, we investigated how modulating copper delivery affected the early crisis seen in SODWTxCCS mice. Our approach was to systematically test a large number of CuATSM chemical derivatives differing in their potential to release copper.

Copper delivery does not necessarily rescue the early death phenotype CuATSM is a planar, neutral, slightly hydrophobic molecule consisting of a bisthiosemicarbazone ligand in complex with a CuII atom (Figure 4). It is proposed that CuII in CuATSM is reduced to CuI and released in cells possessing highly reducing environments resultant from damage to mitochondria (Donnelly et al., 2012). Because CuATSM has an extremely low CuIIàCuI reduction potential relative to biological systems, very little copper is likely to be released *in vivo*. To investigate how modulating copper delivery affected the early crisis seen in SODWTxCCS mice, and to explore the possibility of creating a more effective copper delivery agent, we synthesized a series of bisthiosemicarbazone derivatives with increasing CuIIàCuI reduction potentials. We then systematically tested their efficacy in rescuing the early development crisis seen in SODWTxCCS mice, as measured by copper delivery to the biomarkers COX and SOD.

We first synthesized bisthiosemicarbazones with 2, 1, or 0 methyl groups on the di-imine backbone, called CuATSM, CuPTSM, and CuGTSM, respectively (**Figure 4**). Despite these small structural differences, cyclic voltammetry revealed considerably different reduction potentials of -0.46V, -0.31V, and -0.24V, respectively ***FIG 6***. These reduction potentials predict that the ligated copper in CuATSM is least likely of the three to be chemically reduced (and released) in a biological context, while CuGTSM is the most likely. These reduction potentials agree with reported literature values[JM13] .

We treated SODWTxCCS mice from days 4-15 with CuATSM, CuPTSM, or CuGTSM. At the planned age of 15 days we measured brain COX activity and SOD maturity. Fifteen days was chosen as the primary comparison date because preliminary studies indicated that clear differences are apparent between untreated and CuATSM-treated SODWTxCCS mice at this age, and because 15 days is near-terminal for SODWTxCCS mice without intervention.

CuATSM and CuPTSM treatments significantly increased COX activity and SOD maturity by day 15, compared to untreated control. However, the increase in COX activity and SOD maturity, compared to control, seen in CuGTSM-treated animals was slightly less than that seen in CuATSM- and CuPTSM-treated animals, and was not statistically significant. There were no significant differences in COX activity or SOD maturity between treated groups.

The effects of bisthiocarbazone treatment on COX and SOD were not necessarily reflected in animal weights, for which there were no significant differences. However, each treatment group exhibited a markedly different behavioral phenotype that suggests differential effects on animal health. Untreated and CuGTSM-treated SODWTxCCS pups were immobile and moribund, while CuATSM-treated pups were active and beginning to walk. CuPTSM-treated pups were intermediate in phenotype severity.

Figure4 – Animal weights and copper delivery at 15 days. Treatment began at day 4. CuATSM has a backbone methyl group at R1 and at R2, CuPTSM has one methyl and one hydrogen, and CuGTSM has a hydrogen at R1 and at R2.

Talk about importance of bond length, electron density, reduction potential.

No treatment group weighed significantly more or less than untreated, though CuATSM-treated animals weighed significantly more than CuGTSM-treated animals [ANOVA, $F(3, 34) = 4.333$, p=0.0109, Tukey post-hoc][JM14] .Measurements of COX activity reflect a significant effect of CuATSM- and CuPTSM treatment [ANOVA, $F(3, 30) = 12.48$, $p < 0.0001$, Tukey post-hoc]. Meanwhile, CuGTSM-treated animals were not significantly different from untreated, and no treated group was significantly different from another regarding COX activity. Treatment with CuATSM or CuPTSM had a significant effect on accumulation of mature SOD, but the data for CuGTSM, despite trending similarly to CuATSM and CuPTSM, were too disperse to draw statistical significance from [ANOVA, $F(3, 19) = 4.682$, $p = 0.0130$, Tukey post-hoc].

Given that the early development crisis is seemingly caused by a copper-dependent COX deficiency in the CNS, we hypothesized that all Cu(II)-bisthiosemicarbazones capable of restoring COX activity in the CNS would rescue the early death seen in SODWTxCCS mice. However, our results here suggest there is more to rescuing the early crisis than simply delivering copper to the CNS.

To investigate the possibility that the bisthiosemicarbazone ligand molecules themselves (without copper) have differential, possibly toxic, effects, we treated[JM15] nontransgenic mice with ATSM, PTSM, or GTSM free ligands from 4-21 days and recorded animal weights (Figure 5). GTSM- and PTSM-treated animals weighed significantly less than ATSM- and control-treated animals [ANOVA, $F = 48.68$, $p < 0.0001$, Tukey post-hoc].

Figure5 - Some evidence for differential effects of ligand molecules

[JM16]

Reduction Potentials

[JM17]

Figure 6 - Bisthiosemicarbazone reduction potentials. Talk about biological examples. Show that PhMe derivative(s) was in between CuPTSM and CuGTSM.

Comparison of other compounds

Next we tried other compounds to investigate how other struc-

W851	0.0919	0.0930	0.0925 ± 0.0008

Sample	Rep1 (2015.12.30)	Rep2 (2015.12.30)	Rep3 (2016.02.03)	Avg ± SD
W831	0.0275	0.0254	0.0271	0.0267 ± 0.0011
W832	0.0236	0.0274	0.0247	0.0252 ± 0.0020
W835	0.0250	0.0230	0.0257	0.0246 ± 0.0014
W837	0.0236	0.0265	0.0232	0.0244 ± 0.0018
W838	0.0287	0.0220	0.0257	0.0255 ± 0.0034
W839	0.0226	0.0285	0.0258	0.0256 ± 0.0030

[JM7]There are no statistical outliers (points above or below 1.5 IQR) for SODWTxCCS+CuATSM. Removing the "eyeballed" outliers @ 15d and 21d doesn't change the inflection much – most notably increased 21d SODWTxCCS CuATSM.

[JM8]Include 4do animals

[JM9]4 days

[JM10]be more specific

[JM11]should show in figure: SOD maturity from 15d untreated WTxCCS vs G93AxCCS

[JM12]Include representative mass spectra, deconvolution scheme. Do statistics to compare maturity. Include age-matched G93AxCCS

[JM13]cite

[JM14]Need advice on reporting statistics

[JM15]Mention the blinding and rigor

[JM16]Mock-up.

Maybe go with either the line or the box-and-whiskerrs. Obviously don't include full ANOVA table

Work on caption

Replace "DMSO" with "Control" or "Untreated" to be consistent with the other figures. Can explain in Methods.

[JM17]Placeholder. Will probably have a redox tower (no structures), then a separate structure diagram with substitution labels.

[JM18]Planning on removing individual data points.

Maybe color the "important" groups?

Maybe color the controls individually and use legend to differentiate, label as untreated.

15-day-old data are currently represented with diagonal line pattern.

X labels don't need to be so lengthy.

Remove age.

Straighten labels to 90 deg.

Possibly replace compound labels with key?

W6/

Results

Here we report that WTxCCS mice die early. We then use the WTxCCS mice to investigate how different compounds get Cu in to the CNS.

Homozygous human CCS transgenic mice (described in Williams et al., 2016) were crossed with hemizygous human SODWT transgenic mice (B6SJL-Tg(SOD1)2Gur/J) to produce SODWTxCCS and NTGxCCS offspring. Unlike previous studies, which reported SODWTxCCS mice to have no abnormal phenotype (Son et al., 2008), here we report that our SODWTxCCS mice consistently experience an early development crisis similar to but less severe than high-expressing SODG93AxCCS mice. Our SODWTxCCS mice rapidly developed paralysis and died within three weeks (Figure 1). In six years of breeding, this result has been consistent.

Acknowledgements

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