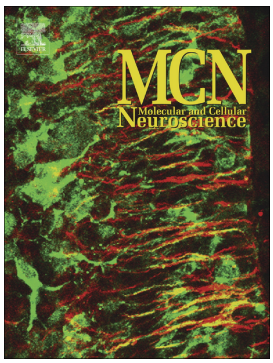

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Assessment of metal concentrations in the SOD1^{G93A} mouse model of amyotrophic lateral sclerosis and its potential role in muscular denervation, with particular focus on muscle tissue

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Abstract

Background: Amyotrophic lateral sclerosis (ALS) is among the most common of the motor neuron diseases, and arguably the most devastating. During the course of this fatal neurodegenerative disorder, motor neurons undergo progressive degeneration. The currently best-understood animal models of ALS are based on the over-expression of mutant isoforms of Cu/Zn superoxide dismutase 1 (SOD1); these indicate that there is a perturbation in metal homeostasis with disease progression. Copper metabolism in particular is affected in the central nervous system (CNS) and muscle tissue.

Methods: This present study assessed previously published and newly gathered concentrations of transition metals (Cu, Zn, Fe and Se) in CNS (brain and spinal cord) and non-CNS (liver, intestine, heart and muscle) tissues from transgenic mice over-expressing the G93A mutant SOD1 isoform ($SOD1^{G93A}$), transgenic mice over-expressing wildtype SOD1 ($SOD1^{WT}$) and non-transgenic controls.

Results: Cu accumulates in non-CNS tissues at pre-symptomatic stages in $SOD1^{G93A}$ tissues. This accumulation represents a potentially pathological feature that cannot solely be explained by the over-expression of mSOD1. As a result of the lack of Cu uptake into the CNS there may be a deficiency of Cu for the over-expressed mutant SOD1 in these tissues. Elevated Cu concentrations in muscle tissue also preceded the onset of symptoms and were found to be pathological and not be the result of SOD1 over-expression.

Conclusions: It is hypothesized that the observed Cu accumulations may represent a pathologic feature of ALS, which may actively contribute to axonal retraction leading to muscular denervation, and possibly significantly contributing to disease pathology. Therefore, it is proposed that the *toxic-gain-of-function* and *dying-back* hypotheses to

explain the molecular drivers of ALS may not be separate, individual processes; rather our data suggests that they are parallel processes.

Keywords: ALS, Copper, Spinal Cord, Brain, Muscle, Distal Motor Neuropathy;

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, which leads to selective motor neuron (MN) death, and is ultimately fatal (Redler and Dokholyan, 2012; Rowland and Shneider, 2001). The degeneration results in progressive muscular paralysis, affecting mobility, speech, and respiration (Hardiman et al., 2011); most patients die within 5 years from diagnosis (Robberecht and Philips, 2013). Most ALS cases are described as sporadic, but around 5 - 10% are familial (Rowland and Shneider, 2001) and associated with a variety of Mendelian-inherited mutations (Robberecht and Philips, 2013).

The first gene identified to be mutated in familial ALS encodes Cu/Zn superoxide dismutase 1 (SOD1), accounting for ~20% of familial ALS cases (Rosen et al., 1993). The specific mechanisms leading to the selective degeneration of MNs remains unclear, with a variety having been proposed to play a role, such as oxidative stress, glutamate-mediated excitotoxicity, protein aggregation and transition metal-induced toxicity (Cozzolino et al., 2008). Toxicity induced by mutant SOD1 (mSOD1) is likely to be the result of a toxic-gain-of-function (Bruijn et al., 1998; Gurney et al., 1994), driven by the increased destabilization of the protein, which causes it to misfold and aggregate. To-date the most studied mouse model of ALS is the transgenic mouse that overexpresses the SOD1-G93A mutation ($SOD1^{G93A}$). In this

model, human SOD1 harboring the G93A mutation is overexpressed (20-24 fold higher expression than endogenous murine SOD1) (Gurney et al., 1994). This mouse model recapitulates many features of human ALS, including axonal and mitochondrial dysfunction, progressive neuromuscular dysfunction, protein aggregation and MN loss (Bruijn et al., 1997; Gurney et al., 1994; Ripps et al., 1995). Results from experimental data suggest that the binding of Cu and Zn by mSOD1 may be defective (Carri et al., 1994; Eum and Kang, 1999; Hayward et al., 2002).

While transition metals (e.g. Cu and Zn) are crucial for the function of many metallo-proteins (e.g. SOD1), they can be toxic at high concentrations (Gaetke and Chow, 2003; Jomova and Valko, 2011; Valko et al., 2005), and their uptake, distribution, storage and excretion are therefore tightly controlled (Hare et al., 2013; Tapiero et al., 2003). The role of metals in the pathogenesis of certain neurodegenerative diseases (i.e. Cu, Fe and Al in Alzheimer's disease (Greenough et al., 2013; House et al., 2012; Kaden et al., 2011; Shore et al., 1984)) is becoming evident; however, the particular role of Cu and Zn homeostasis in the pathology of ALS remains unclear (Roberts et al., 2014).

Findings in the G93A mouse model of ALS indicate that there is a pathologic perturbation in Cu metabolism. While the accumulation and/or depletion of metals in different tissues reported in the literature are inconsistent, overall there appears to be accumulation in tissues of diseased mice. Tissues associated with the autonomic nervous system (ANS) have been shown to accumulate Cu and Zn (Enge et al., 2017; Hilton et al., 2016), with muscle tissue demonstrating pre-symptomatic increases in concentrations (Enge et al., 2017). Results from central nervous system (CNS) tissues

indicate accumulation of Cu in the spinal cord (Li et al., 2006; Tokuda et al., 2015, 2014, 2013, 2008) at single time points, as well as over time (Tokuda et al., 2009, 2007). Brain tissue showed a similarly varied behavior with studies reporting both elevated concentrations (Hilton et al., 2016), or no change to healthy controls (Enge et al., 2017; Lelie et al., 2011; Li et al., 2006; Tokuda et al., 2009, 2007). These changes in Cu and Zn concentrations have been postulated to play an important role in the toxic-gain-of-function associated with mSOD1 (Borchelt et al., 1994; Hilton et al., 2015). Two major strategies to alleviate symptoms of ALS that involve metals have been proposed. These are based on either reducing the general availability of Cu through the application of Cu chelators (i.e. Trietine or *D*-penicillamine), which has been shown to prolong survival of $SOD1^{G93A}$ mice (Hottinger et al., 1997; Nagano et al., 2003); or the application of a synthetic Cu-binding therapeutic agent (Cu^{II}-atsm) to transport Cu across the blood-brain-barrier, which prolongs survival in $SOD1^{G37R}$ and $SOD1^{G93A}$ mice (Hilton et al., 2017; McAllum et al., 2013; Roberts et al., 2014; Soon et al., 2011; Williams et al., 2016).

In SOD1, Cu and Zn ions are crucial for its function: Zn provides structural stability, while Cu is essential for the protein's catalytic activity. In SOD1 mouse models of ALS, mutant SOD1 accumulates in a Cu-deficient form (Lelie et al., 2011; Roberts et al., 2014; Williams et al., 2016). This partially metalated SOD1 is proposed to lead to aggregation and toxicity (Bruijn et al., 1998; Gurney et al., 1994; Roberts et al., 2014). Treatment of mice with the Cu^{II}(atsm) converts the partially metalated SOD1 into holo SOD1 through the delivery of Cu (Roberts et al., 2014), improving the phenotype significantly (Lelie et al., 2011; Roberts et al., 2014; Williams et al., 2016).

This points towards a misbalance of bioavailability and demand for Cu in the CNS (Hilton et al., 2016).

The potential role of metals as well as muscle tissue (Gonzalez de Aguilar et al., 2008; Luo et al., 2013; Xiao et al., 2015) in ALS pathology has recently gathered further attention. In addition to the involvement of metals in binding to SOD1, recent research has suggested that toxic accumulation of metals in muscle tissue may also play a role in ALS pathology (Enge et al., 2017). With pathological hallmarks that include the destruction of the neuromuscular junction (NMJ) and axonal retraction preceding MN death (Zhou et al., 2015), ALS could be described as a distal motor neuropathy (Enge et al., 2017; Fischer et al., 2004; Frey et al., 2000; Kiernan et al., 2011).

In this study concentrations of transition metals in transgenic mutant SOD1 mice (G93A) and non-transgenic controls were assessed. Through the longitudinal comparison of diseased and healthy CNS and non-CNS tissues, this research sought to expand current knowledge on the role and presence of transition metals in the pathology of ALS. ~~Metal accumulations in muscle tissue were assessed for their possible contribution to axonal retraction.~~

METHODS

Samples

This study used two types of transgenic over-expression models, $SOD1^{G93A}$ and $SOD1^{WT}$; only female mice were used in this work. The $SOD1^{G93A}$ mouse model of ALS expressed the G93A mutant form of human SOD1 (B6SJL-

Tg(SOD1*G93A)1Gur/J)(Gurney et al., 1994) (Jackson Laboratory, ME, USA), backcrossed onto a black 6 background. Healthy controls for these mice were non-transgenic littermates (NTG^{UOW}). These mice were bred at Australian Bioresources (Mossvale, AU) in accordance with the approved University of Wollongong ethics clearance (AE14/28). At ages 30, 60, 90 and 120 (±2) days, capturing the characteristic disease progression (Olsen et al., 2001), mice were euthanized by CO_2 asphyxiation and blood collected via puncturing of the left ventricle. The mice were not perfused and following blood collection immediately dissected and tissue samples (brain, spinal cord, liver, intestine, heart and muscle) ~~were~~ snap frozen in liquid nitrogen. Residual blood contamination, due to lack of perfusion is acknowledged and tissues were treated following the principle of 'uniform contribution'. Muscle tissue was samples of *Quadriceps femoris* from both legs and intestine samples were 2 cm sections immediately following the stomach.

The second type of mouse model used was $SOD1^{WT}$ mice, which overexpress the non mutated form of human SOD1 (B6SJL-Tg(SOD1)2Gur/J) (Gurney et al., 1994) (Jackson laboratories). Healthy controls were non-transgenic littermates (NTG^{UCL}). Mice were bred at University College London - Biological Services (London, UK) in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the US National Instituted of Health and under license from the UK Government (Animals and Scientific procedures) Act 1986 (Amended Regulations 2012), following ethical approval from the University College London Institute of Neurology. The samples taken from this mouse model were brain, spinal cord and muscle tissue (*Quadriceps femoris*) samples. These samples were collected at 60 and 90 d to assess if the previously observed increase in Cu concentration in diseased

tissue (Enge et al., 2017) is the result of the over expression of the mSOD1 or presents a pathologic feature, independent of mSOD1 over-expression.

Samples of DORM-2 dogfish muscle, certified reference material of the National Research Council Canada (CRM, NRCC), were used to test the completeness and accuracy of the sample digestion protocol (Enge et al., 2016).

Analytical techniques

Sample digestion

Mouse tissues and DORM-2 aliquots were weighed and pre-digested in MARSXpress 20 mL PFA vessels in a 2.5:1 mixture of 15 M Ultrapur® HNO_3 (Merck) and Ultrapur® 30% H_2O_2 (Merck) for 30 min. Pre-digested samples were completely digested using MARS6 (CEM Corporation, North Carolina, USA) microwave systems. Temperature was ramped to 210 ºC over 15 min and held constant for 150 min to ensure all organic carbon was driven off as gaseous CO_2. For quality control purposes, one blank (acid only) and two DORM-2 aliquots were added to each digestion batch. Recovery of elements (Cu, Fe, Zn and Se) from the DORM-2 certified reference material was used to validate that the digestion of biological samples was complete (Table 1). Selenium concentrations were only determined in liver, intestine and heart tissues.

Table 1 – Recoveries of select metals from DORM-2 (CRM, NRCC), in mg kg^{-1}, during processing of samples

Element	Certified Value	2SD	Measured Value	2SD	Recovery (%)	N
Cu	2.34	0.16	2.4	0.4	104	21
Fe	142	10	133	31	94	21
Zn	25.6	2.3	26	10	109	21

Se	1.40	0.09	1.4	0.2	101	8

Elemental Concentrations

Copper, Fe, Zn and Se concentrations were determined using a Thermo Scientific iCAP-Q quadrupole-inductively coupled plasma-mass spectrometer (Q-ICP-MS) at the Wollongong Isotope Geochronology Laboratory, University of Wollongong (WIGL, UOW). The concentrations were quantified using a multi-element standard external calibration curve; long-term instrument drift was corrected using a 50 ppb Ga solution as an internal standard. Accuracy of the measurements was assessed through the analysis of the DORM-2 CRM, which yielded recoveries of 104% for Cu, 94% for Fe, 109% for Zn and 101% for Se (Table 1). Total procedure blanks were assessed as <4 ng Cu, <9 ng Fe, <18 ng Zn, and <13 ng Se, and deemed negligible (<0.13% for Cu, Zn and Fe; <2% for Se) compared to the total amount processed (see supplementary tables).

Statistical Methods

Statistical analysis was conducted using the statistical program R v3.4.2 (R Core Team, 2016). Prior to analysis, outliers were removed using the median average deviation (3*mad). Any measurements outside 3*mad were considered to be measurement artifacts or the result of contamination. Transgenic SOD^{WT} muscle samples were analysed qualitatively, due to the low sample number. Data were analyzed using linear regression models with disease state and time used as independent variables and Cu, Fe, Zn and Se concentrations, as dependent variables. Likelihood ratio tests were used to determine which independent variables were significant (Barr et al., 2013). The assumptions of the linear regression model were checked (linearity, independence, normality and equality of variance), including

normality tests for residuals. A significance level of $\alpha = 0.05$ was used.

RESULTS

Peripheral tissues

Tissues controlled by the ANS, i.e. the liver, intestine and heart, demonstrate a significant difference in Cu concentration ($p < 0.0005$) between the $SOD1^{G93A}$ and healthy controls (Figure 1A-C), which is evident starting at 30 d, with concentrations in heart and liver increasing over time in the diseased tissues, while they remained relatively constant in the intestine. Zinc concentrations show a similar behaviour to Cu: they vary significantly between $SOD1^{G93A}$ and healthy controls in heart and liver tissue ($p < 0.0005$), and intestine ($p = 0.03$) (Figure 1D-F) starting at 30 d. In heart and liver, the Zn concentrations increase over time, while concentrations in intestine remain largely constant. In contrast to Cu and Zn, Fe concentrations only vary significantly between the diseased and healthy liver ($p = 0.05$) and the intestine ($p = 0.006$) tissue (Figure 2 A-C), and over time in liver ($p < 0.0005$) and intestine ($p = 0.001$). Concerning Se concentrations, only intestine was found to show a significant difference between healthy and diseased tissue ($p = 0.04$), while Se concentrations in heart ($p = 0.002$), liver and intestine ($p = 0.0005$) changed over time (Figure 2D-F).

Muscle tissue (Quadriceps)

As previously shown (Enge et al., 2017), Cu ($p < 0.0005$), Zn ($p < 0.0005$) and Fe ($p = 0.004$) are significantly different between $SOD1^{G93A}$ and NTG^{UOW} mice (Figure 3). Muscle tissues from mice that overexpress the $SOD1^{WT}$ were collected at 60 d and 90 d and tissues from non-transgenic littermates from the same colony were collected at 90 d of age (Figure 4). The concentrations of Cu, Zn and Fe in the SOD^{WT} samples

are in agreement with results from muscle tissue collected from non-transgenic mice from a different colony (NTG^{UOW}) (Enge et al., 2017). The metal concentrations in $SOD1^{WT}$ tissues match well these found in NTG^{UOW} non-transgenic controls (Figure 3).

Central nervous system tissues

It was previously shown that there is no significant difference between brain and spinal cord tissue from $SOD1^{G93A}$ and NTG^{UOW} mice in Cu, Zn and Fe concentrations (Enge et al., 2017). Samples from mice over-expressing $SOD1^{WT}$ and non-transgenic littermates from the same colony (NTG^{UCL}) were collected at 60 and 90 d of age, and are in general agreement with previously presented results of $SOD1^{G93A}$ and NTG^{UOW} mice from a different colony (Supplementary Figure 1).

DISCUSSION

Accumulation of Cu and Zn in tissues controlled by the ANS

Tissues controlled by the ANS play an as yet unknown role in the development of ALS. The accumulation of Cu in the heart and liver could either be pathological and driven by ALS, or the result of the over-expression of SOD1 in the mouse model. As the mouse model of ALS overexpresses (20 - 24 times) human SOD1 harboring the G93A mutation (Gurney et al., 1994), overexpression of the protein could result in it acting as a Cu sink (Tokuda and Furukawa, 2016). However, if this were the case a uniform offset with regards to the Cu concentration in tissues from $SOD1^{G93A}$ mice compared to the non-transgenic mice would be expected (similar to that observed for the intestine where concentrations did not change significantly over time). A lack of increases of SOD1 concentration with time in spinal cord and brain of various mouse

models of ALS was observed and attributed to reflecting high-level relative steady-states (Jonsson et al., 2006); though, work by Turner et al. (2003) has shown age-dependent accumulation of hSOD1 in the lumbar spinal cord, the sciatic nerve, and the gastrocnemius muscle. In our work (Enge et al., 2017) it was acknowledged that differences in Cu and Zn concentration between the $SOD1^{G93A}$ and non-transgenic mice in muscle tissue could be due to over-expression of human SOD1. Here, observations of a lack of elevated concentrations in Fe and Se (Figure 2) point towards a great contribution of over-expressed SOD1 to the Cu accumulation. This accumulation was also reported in previous work (Tokuda et al., 2013): in spinal cord tissue of $SOD1^{WT}$ mice (over-expression of wild-type SOD1) and $SOD1^{G93A}$ mice Cu concentrations were elevated (up to 3.2-fold) compared to non-transgenic controls. Furthermore, Cu concentrations in spinal cord were significantly elevated in $SOD1^{G93A}$ samples compared to $SOD1^{WT}$ samples (Tokuda, 2017). This indicates that at least part, if not all of the accumulation of Cu and Zn in these tissues could be attributable to SOD1 over-expression. However, the distinct accumulation of Cu in heart and liver over time (Figure 1) compared to controls may represent disease pathology: previously it was found that in $SOD1^{G93A}$ spinal cord tissue, Cu was bound to copper-binding proteins other than SOD1 and this increased over time (Tokuda et al., 2013). Accumulation of Cu in cupro-proteins besides SOD1 may therefore contribute to the observed signal. Accumulation of Cu in cupro-proteins besides SOD1 may therefore contribute to the observed signal. The observation of a subtle age-dependent increase of hSOD1 in the gastrocnemius muscle (Turner et al., 2003) could indicate that a relative portion of the observed metal accumulation is a result of SOD1 accumulation with age. The lack of SOD1 concentration measurements here presents a limitation to the conclusions that can be drawn: without tissue-matched

measurements from mice that overexpress $SOD1^{WT}$, or determination of SOD1 concentrations with age, estimation of how much of the observed increase in Cu concentration is attributable to the over-expression of SOD1 in peripheral system tissues remains uncertain.

Zinc concentrations were found to be significantly elevated in all three tissues (liver, heart and intestine) and their concentrations in heart and liver increase over time (Figure 1D-F). Similar to Cu, increased concentration of Zn in heart and liver of $SOD1^{G93A}$ mice are likely to be related to the over-expression of SOD1, as other elements (Fe and Se) were not increased (Figure 2). Another factor may also contribute: work using spinal cord tissue from $SOD1^{G93A}$ mice showed an accumulation of Zn not only in the zinc-binding site of SOD1, but also suggested an increase of Zn binding in other Zn binding proteins (Tokuda et al., 2013). The increase in Zn concentration was also statistically significant in the intestine; the difference is lower and relatively constant over time.

While the accumulation of Cu and Zn in these tissues precede disease pathology and symptoms, coinciding with initial protein aggregation at 30 d (Gould et al., 2006) (Table 2), the specific binding of the metal(s) to SOD1 has to be further scrutinized.

Table 2 – Typical ALS symptoms and features in transgenic SOD1 mice

Feature/symptom	**Average time of onset (d)**	**Reference**
Initial protein aggregation	30	(Gould et al., 2006)
Selective neuromuscular junction degeneration	47	(Fischer et al., 2004)
Maximum running speed reduced	52	(Veldink et al., 2003)
Reduction of blood flow through the spinal cord	60	(Zhong et al., 2008)

Axonal loss prominent	80	(Fischer et al., 2004)
Significant motor neuron loss	100	(Fischer et al., 2004; Seki et al., 2007)
Onset of paralysis	100	(Chiu et al., 1995)

Lack of up-regulation of Cu transport into CNS

Previously no increase in Cu and Zn concentrations was found in the CNS (Enge et al., 2017); in this work we show these do increase in non-CNS tissues (Figure 1). This could be the result of the non-uniform expression of mSOD1 in various tissues. Results in humans (Human Protein Atlas, 2017) as well as in the $SOD1^{G37R}$ (Hilton et al., 2016) and $SOD1^{G93A}$ (Gajowiak et al., 2016) mouse models have shown that SOD1 protein expression varies between tissues; $SOD1^{G37R}$ CNS tissues expressed much greater amounts of SOD1 compared to peripheral nervous system tissues (Hilton et al., 2016). In tissues from $SOD1^{G93A}$ mice, higher amounts of SOD1 were reported in spinal cord compared to liver and muscle (*Gastrocnemius*) (Gajowiak et al., 2016). Thus, if Cu and Zn concentrations reflected the amount of SOD1 present, it would be expected that CNS tissues would have higher concentrations of Cu and Zn compared to other tissues. The origin for the discrepancy in Cu and Zn concentrations between CNS and non-CNS tissues is therefore more likely to be the response of the blood-CNS barriers. While an increased Cu requirement in the CNS, due to the SOD1 over-expression, may not be met (Hilton et al., 2016), it is unclear why Zn concentrations are not elevated.

Our results show an accumulation of Cu and other metals in the heart and liver tissue compared to the CNS, as well as compared to non-transgenic controls (Enge et al., 2017; Hilton et al., 2016). Blood of ALS patients was shown to not accumulate Cu

and Zn, compared to controls (Garzillo et al., 2014; Kapaki et al., 1997; Nagata et al., 1985; Pamphlett et al., 2001; Roos et al., 2013). This agrees with our previous findings in the $SOD1^{G93A}$ mouse (Enge et al., 2017). Blood carries a large amount of the total body Cu, which can be regarded as a theoretically infinite pool for the individual compartments. The observed increases in concentration in tissues such as heart and liver in the $SOD1^{G93A}$ mice, despite no change in blood (Enge et al., 2017), suggests that acquisition of Cu from the liver into the blood does not vary. Overall the increased demand for Cu in the organism due to higher mSOD1 levels in these tissues is satisfied. Copper concentrations in the heart, liver and intestine may therefore serve as an additional proxy for disease in the $SOD1^{G93A}$ mouse model of ALS.

The lack of an increase in Cu concentration in the CNS may reflect a disconnect between the rate of Cu uptake and Cu requirement (Hilton et al., 2016) as a response to the over-expression of mSOD1. Recent results have shown that the amount of Cu in both brain and spinal cord tissue of the $SOD1^{G37R}$ mouse model is disproportionately small compared to the amount of SOD1 protein expressed (Hilton et al., 2016). This indicates that as well as a generally slower turnover, Cu transport into the CNS is not up-regulated to satisfy the increased Cu requirement due to mSOD1 over-expression. This effectively 'starves' the over-expressed mSOD1 (Figure 4) (Hilton et al., 2016). As a result, the protein could be destabilized, making it aggregation-prone (Lelie et al., 2011). The result could be MN death through direct toxicity of the partially metalated SOD1 (Gil-Bea et al., 2017; Roberts et al., 2014).

Pathological accumulation of Cu in muscle tissue may contribute to ALS development

Muscle tissue (Gonzalez de Aguilar et al., 2008; Luo et al., 2013; Xiao et al., 2015), and other (neighbouring) cell types (Boillée et al., 2006) may play a significant role in the development of ALS pathology. Distinct and rapid muscle atrophy caused by MN death is a pathologic feature of ALS. Skeletal muscle comprises ~40% of whole body lean mass and, combined with bone, makes up ~50% of total body Cu in humans (Evans, 1973). We previously showed consistently elevated metal concentrations in $SOD1^{G93A}$ muscle tissue compared to samples from non-transgenic animals (Enge et al., 2017), in accordance with previous findings (Hilton et al., 2016). These increases of Cu and Zn concentrations precede the onset of disease symptoms (Figure 3A-B), while Fe trails them (Figure 3C) (Enge et al., 2017). We hypothesized that this observed accumulation could be the result of the over-expression of mSOD1 in the $SOD1^{G93A}$ mouse model and that further testing, involving tissues from mice that over-express $SOD1^{WT}$ was necessary to determine whether a pathologic accumulation of Cu occurred in muscle tissue.

Here, Cu, Zn and Fe concentrations measured in samples from mice over-expressing wild type SOD1 were qualitatively assessed due to low available sample numbers, and found to be ~~were~~ similar to those ~~found~~ in tissues from non-transgenic controls (Figure 3A-C). This is in agreement with results from a previous study comparing Cu in spinal cord tissues from mice over-expressing wild-type SOD1 and non-transgenic controls (Tokuda et al., 2009). Since the accumulation of these metals in muscle tissue is not related to SOD1 over-expression, we contend that it represents a pathological feature of ALS that warrants further investigation, particularly as the accumulation of Cu is pre-symptomatic (Figure 3A). While the method applied here does not enable us

to determine the origin of the accumulation, several hypotheses regarding its involvement in ALS pathology are presented below:

First, as previously proposed (Enge et al., 2017), Cu accumulation may result in toxicity at the NMJ and the onset of ALS as a distal motor neuropathy that proceeds via a 'dying-back' mechanism (Enge et al., 2017; Fischer et al., 2004; Frey et al., 2000; Kiernan et al., 2011). In this case distal axonal degeneration precedes neuronal degeneration, whereby axons and NMJs are affected early and the MN withdraws due to perturbations arising from muscle tissue (Zhou et al., 2015). This contrasts the *toxic-gain-of-function* and '*dying-forward*' hypotheses in which denervation results from the CNS. To understand the potential role of muscular Cu as a trigger for axonal denervation, its location within the tissue must be considered: Cu may reside in the cytosol or extracellular space. In the cytosol, it could lead to increased reactive oxygen species (ROS)-related stress and ultimately cellular damage and apoptosis (Linder, 1991). However, Cu is unlikely to be present as a free ion, as this would trigger an anti-oxidant response through SOD1 and other protective mechanisms, in order to reduce its impact. Mechanisms to protect against Cu toxicity include the intrinsic stress response of the heat shock proteins (e.g. heat shock protein 70) (Urani et al., 2001), which can be triggered by sub-lethal concentrations of Cu. Additionally, the metal-responsive transcription factor 1 (MTF-1) (Balamurugan and Schaffner, 2006) acts under both high and low Cu concentrations to control the expression of metallothioneins and other components (Cu importer/transporter 2) that control Cu homeostasis. Copper in the extracellular space can bind to a variety of proteins, including ceruloplasmin, extracellular SOD1, extracellular metallothionein and albumin (Linder and Hazegh-Azam, 1996). The work presented here is limited to

organ-level resolution and therefore does not provide any insight into how Cu accumulates in tissues, including whether it is located inside or outside cells, or both.

Second, the accumulation of Cu in muscle tissue may be the consequence of hypermetabolism. Increases in energy expenditure are intrinsically linked to ALS progression, preceding axonal retraction and denervation (Dupuis et al., 2004; Dupuis and Loeffler, 2009; Ferri and Coccurello, 2017). Even though previous work has shown that mitochondrial defects precede the onset of MN loss (Basun et al., 1991; Jaarsma et al., 2000; Kong and Xu, 1998), specific mechanisms and causal links between hypermetabolism and mitochondrial dysfunction remain largely unknown.

Mitochondrial respiration produces electrons, which may escape the electron transport chain to induce the formation of reactive oxygen species (ROS) (Adam-Vizi and Chinopoulos, 2006; Liemburg-Apers et al., 2015; Murphy, 2009). Mitochondrial dysfunction, including muscle tissue, has been identified as one of the key features of ALS (Leclerc et al., 2001); aggregated, swollen, vacuolated and fragmented mitochondria, as a possible result of mSOD1 interaction (Pickles et al., 2016), are able to explain other pathological features such as oxidative stress, glutamate excitotoxicity and apoptosis (Abel et al., 2012; Faes and Callewaert, 2011; Martin, 2011; Smith et al., 2017). It was suggested that dys-regulation of Cu homeostasis may play a substantial role in the development of the mitochondrial defects associated with ALS (Son et al., 2007).

The observed Cu accumulation in muscle tissue may be the result of more than one mechanism, whereby the initial increase could result from an unidentified process that also increases ROS production. Increases in ROS could stimulate glucose uptake, which in return could stimulate ROS production leading to a positive feedback loop (Liemburg-Apers et al., 2015). Simultaneously, Cu transport into mitochondria could be up regulated to meet the demand of cellular respiration, resulting in the observed hypermetabolism and overall accumulation of Cu in the tissue.

An updated view on the role of Cu in muscle tissue in ALS

The specific role of accumulated Cu in muscle tissue associated with ALS pathology remains unclear. The data presented here shows an increase in metal concentrations in muscle tissues of the $SOD1^{G93A}$ mouse model over time. This is interpreted as a pathological process (Figure 5C), which may result in both a *toxic-gain-of-function* (partially metalated SOD1 aggregates are toxic and lead to axonal retraction), as well as a *dying-back* mechanism (e.g. accumulation of insoluble mSOD1 aggregates and glutamate toxicity result in ALS presenting as a distal axonal retraction that precedes death of MNs [see Ref (Dadon-Nachum et al., 2011) for a comprehensive review]) of ALS onset and progression. The unifying feature in both these mechanisms is Cu, be it the above-described lack of up-regulation in the CNS or the apparent accumulation in ANS tissues. We therefore propose a new theoretical model here, in which both the *toxic-gain-of-function* and the *dying-back* hypotheses could be occurring in parallel, to explain the mechanisms of ALS pathology (Figure 5C). The relative proportion to which each process contributes to the end result of muscular denervation remains unclear.

The model proposed here combines findings of general Cu accumulation in peripheral tissues, specifically muscle tissue, and lack of up-regulation of Cu in the CNS. It is proposed that there are separate processes (Figure 5A, B) associated with Cu (either as a trigger or consequence), whose specific distribution on an organ level we have tested (Figure 5C). This specific distribution may contribute to the development of ALS in these mice (see above). Furthermore, the model explains why Cu chelators (Hottinger et al., 1997; Nagano et al., 2003), as well as synthetic Cu-binding therapeutic agents (Roberts et al., 2014) likely act to prolong the survival of SOD1 mouse models. In addition to the transformation of labile intermediate forms of SOD1 into apo-SOD1, (Roberts et al., 2014) Cu chelators may also remove some generally available Cu to prevent its accumulation in tissues. Simultaneously, Cu^{II}(atsm), which is able to readily cross the BBB, was found to deliver Cu to mSOD1 so it is in a (stable) holo-SOD1 state (Hilton et al., 2017; Soon et al., 2011; Williams et al., 2016). Prolonged survival (>20%) in G93A mice treated with Cu^{II}(atsm) has been reported, however this lifespan is greatly extended in G93A mice co-expressing the copper chaperone for superoxide dismutase (CCS) (G93AxCCS) and treated transdermally from prenatal stages (Williams et al., 2016). Even though this presents a very important milestone in finding a feasible treatment option for ALS, it is not a cure as all mice developed typical end stage motor neuron disease. Furthermore, G93AxCCS mice removed from treatment after 21 days had the same life span as $SOD1^{G93A}$ mice (Williams et al., 2016). This indicates that at least part of the toxic-gain-of-function of SOD1 resides in mSOD1 being metal deficient, while pathological Cu accumulation in muscle tissue may also contribute.

Based on our work and the model we have proposed here, a treatment combining a specific Cu chelator (to prevent Cu accumulation in non-CNS tissues), and a synthetic Cu-binding therapeutic agent (to deliver Cu to the CNS) could be most beneficial to treat ALS. However, since some studies using human samples did not observe Cu accumulation in neither liver or kidneys (Sillevis Smitt et al., 1992), nor in brain (Gellein et al., 2003) of ALS patients compared to controls, the therapeutic potential of such a treatment remains elusive. Moreover, data showing the lack of general accumulation of Cu in human ALS patients is non-conclusive at this point due to the overall limited number of studies available. Future studies should systematically assess Cu concentrations in human ALS patient organs, including muscle tissues, target tissues of ALS. Furthermore, Cu accumulation in ALS models has to further be examined as it may serve as a biomarker of disease.

CONCLUSIONS

The assessment of metal concentrations in several tissues from control and ALS mice showed accumulation of Cu in tissues controlled by the ANS compared to controls, which was also not observed in CNS tissues. This accumulation was identified to be pre-symptomatic. Muscle tissue showed accumulation of Cu, which was not driven by over-expression of SOD1 (Enge et al., 2017), but rather was associated with disease pathology in $SOD1^{G93A}$ mice and was evident at pre-symptomatic stages. The results further indicate that there is a lack of Cu uptake into the brain despite the higher Cu requirement due to mSOD1 over-expression in the $SOD1^{G93A}$ mouse model of ALS. The observed pre-symptomatic changes in metal concentrations in tissues controlled by the ANS provide further evidence for a role of metals, in particular Cu, in the development of ALS pathology.

The accumulation of Cu in muscle tissue leads us to propose a revised model of the mechanisms underpinning ALS pathology, which unites the *toxic-gain-of-function* and *dying-back* hypotheses. Our model offers another explanation to elucidate the axonal retraction of MNs in ALS, a process potentially underpinned by Cu accumulation. Further investigation into the potential applicability of metal concentrations as biomarkers for ALS is therefore warranted. Future studies should expand to study human tissues, and further establish in disease models the location and binding of Cu in ANS tissues to help further elucidate the role that Cu plays in the aetiology of ALS.

LIST OF ABBREVIATIONS

Ab	Albumin
ALS	Amyotrophic lateral sclerosis
ANS	Autonomic nervous system
ATOX	Copper metal chaperone
ATP7A	ATPase copper transporting alpha
BBB	Blood-brain-barrier
BCB	Blood-cerebrospinal fluid-barrier
CCS	Copper chaperone for superoxide dismutase
CNS	Central nervous system
COX	Cytochrome *c* oxidase
CP	Ceruloplasmin
CTR1	Copper transporter 1
CTR2	Copper transporter 2
Cu^{II}(atsm)	Diacetyl-bis(4-methylthiosemicarbazonato)$copper^{II}$
DMT1	Divalent metal transporter 1
FUS	Fused in Sarcoma
MN	Motor neuron
MTF1	Metal regulatory transcription factor 1
NMJ	Neuromuscular junction
Q-ICP-MS	Quadrupole-inductively coupled plasma-mass spectrometer
ROS	Reactive oxygen species
SOD1	Cu,Zn superoxide dismutase 1
TDP-43	TAR DNA-binding protein 43

DECLARATIONS

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Authors' contributions: TGE and HE collected the UOW-based samples. BK collected the UCL-based samples. TGE processed and analyzed the samples. TGE, HE, DFJ and AD interpreted the data and conceived the manuscript. TGE, HE, DFJ, JJY, BK and AD contributed to the editing of the manuscript. All authors read and approved the final manuscript.

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Supplementary material:

File Name: Supplementary Material_Data
File Format: .docx
Title: Raw data
Description: This data set includes data relevant to this paper.

File Name: Supplementary Material_Figure 1
File Format: .docx
Title: Supplementary Figure 1
Description: This Figure presents Cu and Zn concentrations (µg g^{-1} of dry tissue) in brain and spinal cord tissue as a function of time (days). This includes previously published results and newly produced results.

Figure 1: Copper (A-C), Zn (D-F) concentrations in tissues controlled by the ANS (heart, liver and intestine) (µg g^{-1} of dry tissue) as a function of time (days). All data are presented as box (median ±95% CI) and whisker (maximum and minimum) plots ($n \geq 5$).

Figure 2: Iron (A-C), Se (D-F) concentrations in peripheral tissues (heart, liver and intestine) (µg g^{-1} of dry tissue) as a function of time (days). All data are presented as box (median ±95% CI) and whisker (maximum and minimum) plots ($n \geq 5$).

Figure 3: Copper, Zn and Fe concentrations (µg g^{-1} of dry tissue) in muscle tissue as a function of time (days). Boxplots (median ±95% CI) show values for $SOD1^{G93A}$ mice and non-transgenic littermates ($n \geq 5$); circles indicate outliers. Triangles and circles depict additional samples of $SOD1^{WT}$ mice and non-transgenic littermates (see legend). Se was not measured in these samples.

Figure 4: Generalized model of Cu transport by the blood-brain-barrier (BBB) and blood-cerebrospinal fluid-barrier (BCB). Free Cu ions are transported, predominantly via the BBB, into the CNS, where they distribute following a concentration gradient. The removal of Cu from the CNS is currently not well understood but appears to occur via the BCB. In the $SOD1^{G93A}$ mouse model of ALS, a general accumulation of Cu is observed in peripheral nervous system tissues in response to the over expression of mSOD1. Besides this increase, no other compartment demonstrates an increase in Cu concentration, indicating that there is no up regulation of Cu transport from blood into the central nervous system, resulting in 'starvation' of the over-expressed mSOD1. Modified from Zheng and Monnot, 2012 (Zheng and Monnot, 2012).

Figure 5: Generalised and simplified schematic of pathological processes in ALS. (A) Dying forward hypothesis: Along side mitochondrial dysfunction and overall increased oxidative stress, demetalated mSOD1, lacking Cu, aggregates (1) and results in a toxic-gain-of-function (2), which leads to axonal retraction and denervation (3). (B) Dying back hypothesis: (1) mitochondrial dysfunction, glutamate toxicity, accumulation of insoluble mSOD1 and neurofilaments, as well as selective neuromuscular synapsis vulnerability, lead to (2) pathological changes in nerve terminals and axonal retraction, resulting (3) in muscular denervation.
In a revised model, including the latest findings of Cu concentrations in the $SOD1^{G93A}$ mouse model (C), it is proposed that besides the toxic gain-of-function (2), accumulation of Cu in muscle tissue contributes to axonal retraction (2) either via direct toxicity or a more complex mechanisms (e.g. oxidative stress-induced mitochondrial dysfunction), leading to (3) muscular denervation. Possible intervention options including Cu chelators to remove excessive Cu from muscle tissue and Cu chaperones to deliver Cu to SOD1, are also highlighted.

Highlights

- Metals accumulate pre-symptomatically in non-CNS tissues in SOD1^{G93A} ALS mouse model
- Pathological Cu concentrations in muscle tissue precede onset of symptoms
- Tissue specific accumulation of Cu could indicate a traceable, pathological feature
- New model proposes significant role of Cu in the development of ALS

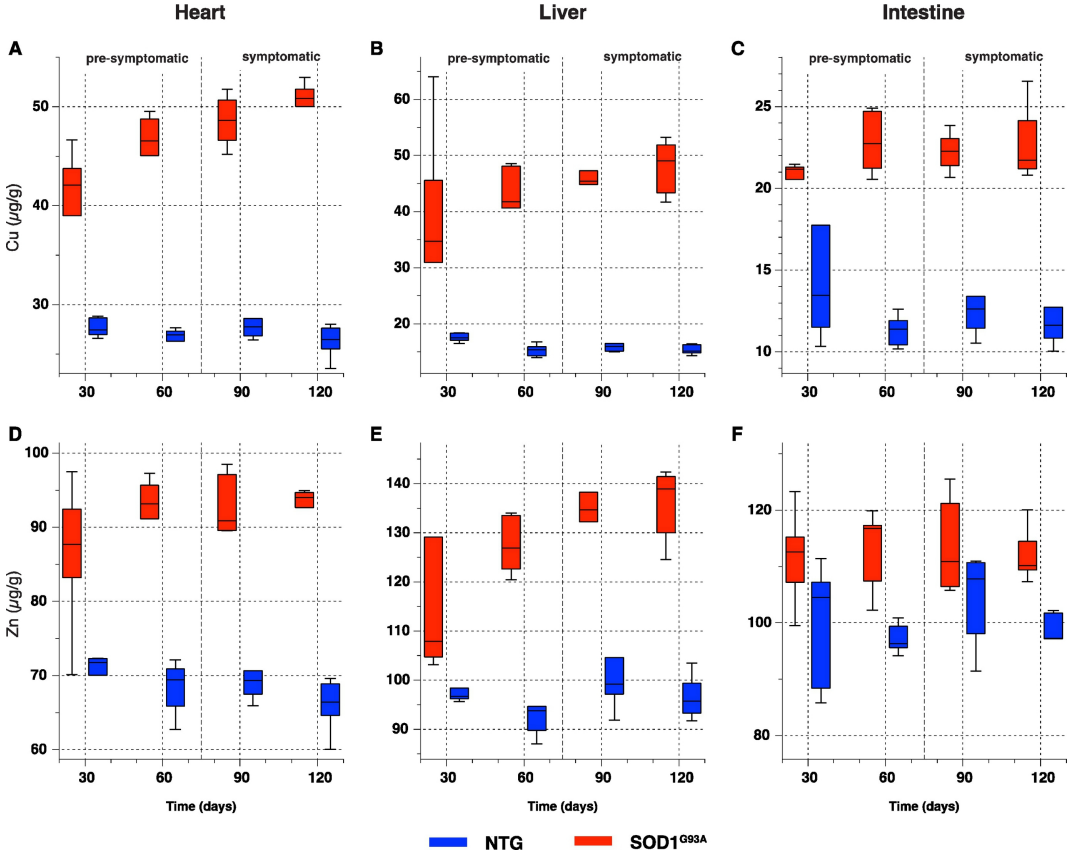
Heart
Liver
Intestine
A
B
C
D
E
F
pre-symptomatic
symptomatic
Cu (μg/g)
Zn (μg/g)
Time (days)
NTG
SOD1G93A

Figure 1

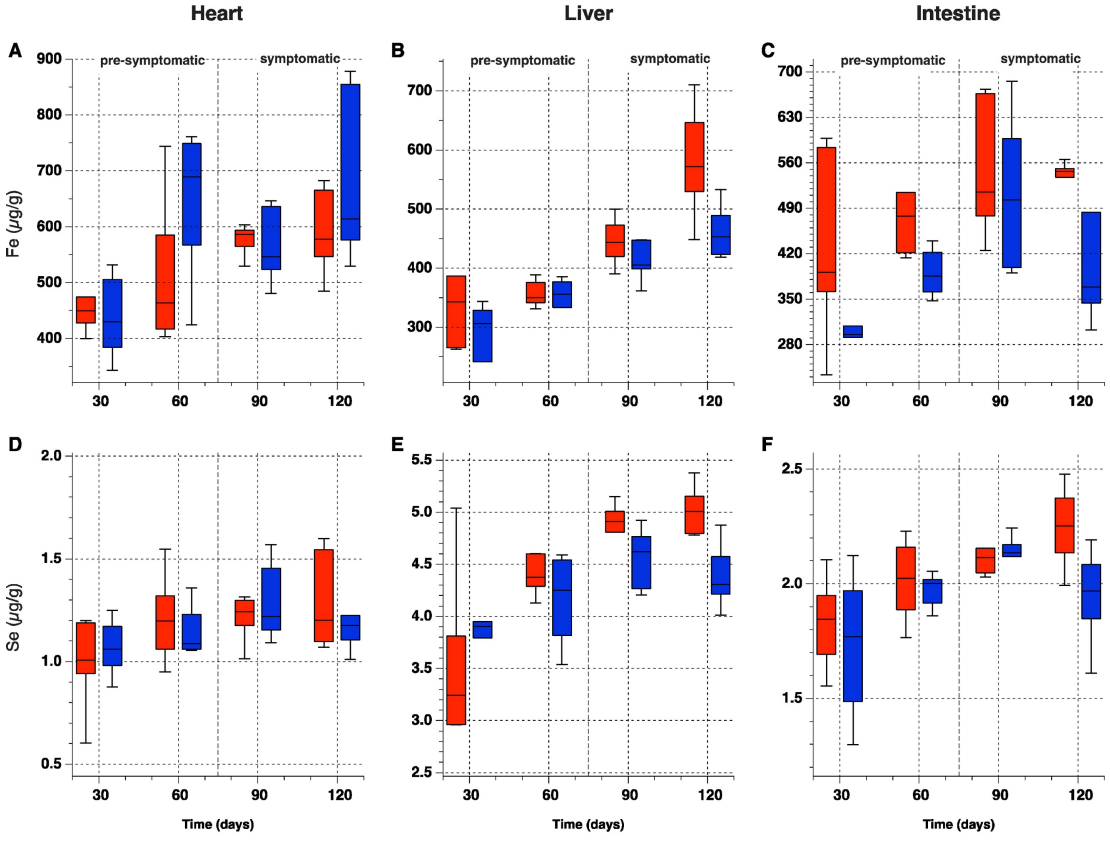
Heart
Liver
Intestine
A
B
C
D
E
F
pre-symptomatic
symptomatic
Fe (μg/g)
Se (μg/g)
Time (days)
NTG
SOD1G93A

Figure 2

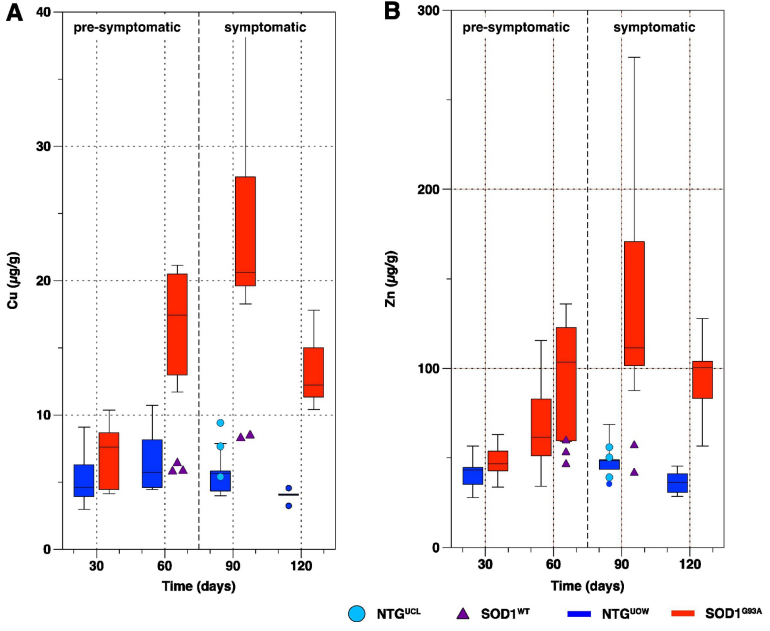
A
pre-symptomatic
symptomatic
Cu (µg/g)
40
30
20
10
0
30
60
90
120
Time (days)
B
pre-symptomatic
symptomatic
Zn (µg/g)
300
200
100
0
30
60
90
120
Time (days)
C
pre-symptomatic
symptomatic
Fe (µg/g)
200
100
0
30
60
90
120
Time (days)
NTG^UCL
SOD1^WT
NTG^UOW
SOD1^G93A

Figure 3

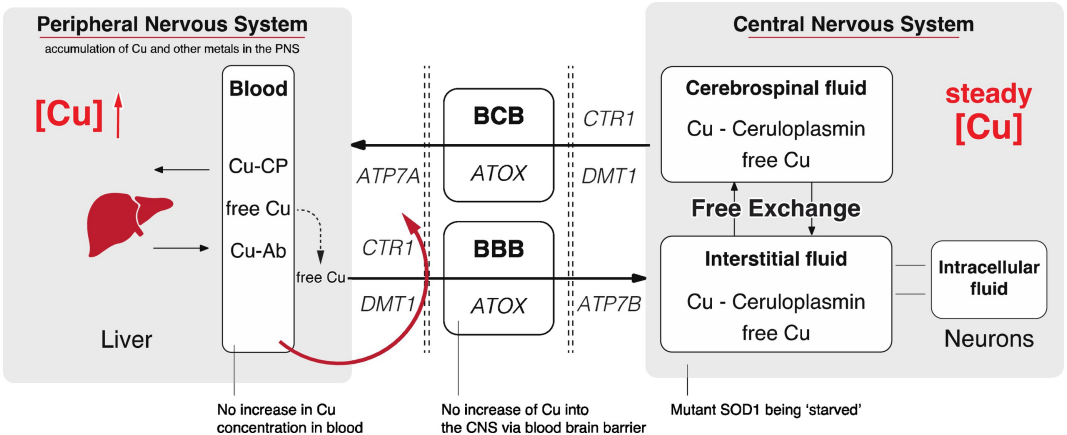
Peripheral Nervous System
accumulation of Cu and other metals in the PNS
[Cu]
Blood
Cu-CP
free Cu
Cu-Ab
Liver
free Cu
ATP7A
CTR1
DMT1
BCB
ATOX
BBB
ATOX
CTR1
DMT1
ATP7B
Central Nervous System
Cerebrospinal fluid
Cu - Ceruloplasmin
free Cu
steady
[Cu]
Free Exchange
Interstitial fluid
Cu - Ceruloplasmin
free Cu
Intracellular fluid
Neurons
No increase in Cu concentration in blood
No increase of Cu into the CNS via blood brain barrier
Mutant SOD1 being 'starved'

Figure 4

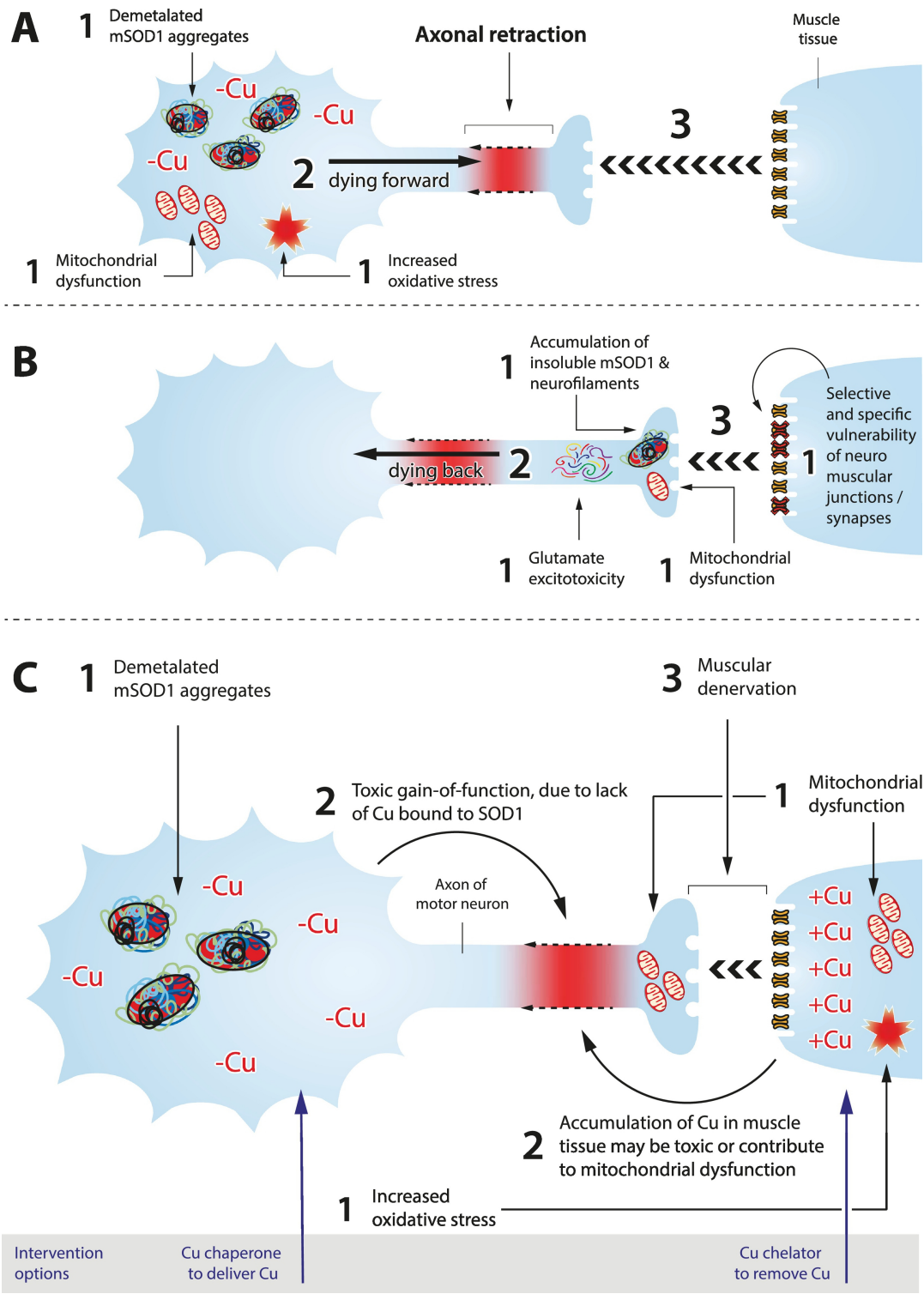
A
1 Demetalated mSOD1 aggregates
Axonal retraction
Muscle tissue
-Cu
-Cu
-Cu
2 dying forward
3
1 Mitochondrial dysfunction
1 Increased oxidative stress
B
1 Accumulation of insoluble mSOD1 & neurofilaments
Selective and specific vulnerability of neuro muscular junctions / synapses
3
dying back
2
1
1 Glutamate excitotoxicity
1 Mitochondrial dysfunction
C
1 Demetalated mSOD1 aggregates
3 Muscular denervation
2 Toxic gain-of-function, due to lack of Cu bound to SOD1
1 Mitochondrial dysfunction
Axon of motor neuron
-Cu
-Cu
-Cu
-Cu
-Cu
+Cu
+Cu
+Cu
+Cu
+Cu
2 Accumulation of Cu in muscle tissue may be toxic or contribute to mitochondrial dysfunction
1 Increased oxidative stress
Intervention options
Cu chaperone to deliver Cu
Cu chelator to remove Cu

Figure 5