Examining the Human Relevance of a New Mouse Model of ALS and Observations on the Incidence in Human Populations

by
Lindsay Winkenbach

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biochemistry and Biophysics
(Honors Associate)

Honors Baccalaureate of Arts in International Studies
(Honors Associate)

Presented May 12, 2016
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AN ABSTRACT OF THE THESIS OF

Lindsay Winkenbach for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics and Honors Baccalaureate of Arts in International Studies presented on May 12, 2016.
Title: Examining the Human Relevance of a New Mouse Model of ALS and Observations on the Incidence in Human Populations

Abstract approved:

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Joseph Beckman

Amyotrophic Lateral Sclerosis (ALS) is a relatively rare neurodegenerative disorder that is fatal 2-4 years after the onset of the disease. Population studies are being done in order to help reveal candidates for further studies of disease etiology, disease progression, as well as to further understand racial and ethnic variations. Population-based studies have occurred mostly in the United States and Europe, with few to no studies conducted in other regions. One of the most widely used ALS animal models is the SOD^{G93A} mouse model. Up until now there has been no effective treatment significantly expanding the lifespan of this model. The Beckman lab has demonstrated that the copper ligand CuATSM effectively halts the progression of ALS symptoms in a variant of the SOD^{G93A} mouse model co- expressing the Human Copper Chaperone for SOD, hCCS. However, this SOD^{G93A}xhCCS mouse model is not well-characterized in terms of relative and absolute protein expression levels of SOD compared to CCS. The research done for this thesis aimed to fill this knowledge gap. We demonstrated that the relative expression ratio of SOD1 to hCCS in the SOD^{G93A}xhCCS mouse model is 1.5:1. These preliminary results will provide insights when comparing this new mouse model to humans.

Key Words: Amyotrophic Lateral Sclerosis (ALS), SOD^{G93A} mouse model, CuATSM, SOD1, hCCS

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Honors Baccalaureate of Arts in Biochemistry and Biophysics and Honors Baccalaureate of Arts in International Studies project of Lindsay Winkenbach presented on May 12, 2016.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

Lindsay Winkenbach, Author

Chapter 1: ALS History and Epidemiology

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive, uniformly fatal motor neuron disease (MND) characterized by degenerative changes to motor neurons[1–4]. Patients experience signs and symptoms of progressive muscle atrophy and weakness, increased fatigue and problems with swallowing, which typically lead to respiratory failure and death[4]. Progressive functional deficits lead to paralysis and death occurs within 2–4 years from onset. Only 5–10% of patients survive beyond 10 years[3]. The pathology is characterized by a late age of onset (50-60 years) with a slight male prevalence (male: female ratio 3:2)[5]. Because of the rapid death rate, the incidence of ALS is higher than the number of afflicted patients compared to other diseases, such as Multiple Sclerosis.

French neurobiologist and clinician Jean-Martin Charcot was the first to describe ALS over 135 years ago based on connections made between clinical signs and the findings at autopsy. Initially called Charcot's sclerosis, the current name is derived from Charcot's observation of degeneration and death of the upper and lower motor neurons in the lateral portions of the spinal cord, leading to fatal paralysis[6]. The term "amyotrophic" refers to the loss of muscle mass due to degeneration and loss of axons of upper motor neurons, "lateral" defines the sites where neurons undergo degeneration along the tract where nerves run down both sides of the spinal cord, and "sclerosis" describes the appearance of tissue along the spinal cord after degeneration, which appears scar tissue like in nature[7].

The disease is more colloquially known now as Lou Gehrig's disease, named after the famous Yankees baseball player whose impressive career came to an end

due to ALS in 1941. This began to bring much more awareness to the disease and his name now is a source of inspiration and courage to many living with the disease[8]. In 2014 the ALS association introduced the Ice Bucket Challenge to raise funds and awareness, ultimately raising $115 million dollars[9].

ALS POPULATION STUDIES

The World Federation of Neurology Research Committee has developed a method for diagnosis of ALS, called the El Escorial criteria (Table 1-A)[10]. However, when these criteria were applied rigidly, 40% of the ALS patients were excluded from enrollment in clinical trials at the time where symptoms were presenting[11]. In 2013 the Pitie- Salpetriere Hospital in France updated the diagnostic criteria that takes a more stepwise approach to diagnosis that ranges from clinically possible to clinically definite (Table 1-B)[1]. Because diagnostic criteria require expensive technology, such as MRIs, population data can be difficult to meet in countries lacking access to these machines.

Table 1-A: Diagnostic criteria as described by the World Federation of Neurology Research

Symptoms Present	Symptoms Absent
Evidence of lower motor neuron (LMN) degeneration by clinical, electrophysiological or neuropathologic examination	Electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration
Evidence of upper motor neuron (UMN) degeneration by clinical examination	Neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.
Progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination	

Table 1-B: Revised El Escorial Diagnostic Criteria for ALS

Clinically Possible ALS	Laboratory Supported Probable ALS	Clinically Probable ALS	Clinically Definite ALS
UMN and LMN signs in one region, or UMN signs in at least two regions, or UMN and LMN signs in two regions with no UMN signs rostral to LMN signs	UMN signs in one or more regions and LMN signs defined by electromyogram in at least two regions	UMN and LMN signs in two regions with some UMN signs rostral to the LMN signs	UMN and LMN signs in three regions

Another important tool for conducting population-based studies are population-based registries, which are used in order to detect all cases of a specific disease, such as ALS, defined by geographic boundaries[3]. Data from population-based registries greatly contributes to clinical knowledge as well as epidemiologic studies to better understand population specific occurrences. These registries have the capability to be incredibly comprehensive by conducting large-scale studies that take into account both environmental and genetic risk factors. However, many groups of people have the potential to be left out. Within a population that has a registry, it is possible that the surveillance system will not include the extreme elderly as well as very low-income groups. On a more international level, this approach of a population-based registry becomes only available in countries with the means of collecting these data, leading to very limited population data in many regions.

Population-based studies have taken place mostly in the United States and Europe, with fewer studies conducted in the southern hemisphere. In a systematic review conducted by the National Institute on Aging and National Institutes of Health, a comprehensive search was done to compile observational studies on the epidemiology of ALS, specifically looking at data on incidence and prevalence

between distinct populations (Figure 1). Most of the data came from Europe, with Italy best represented at eleven studies. Only two studies were conducted within the southern hemisphere- one in New Zealand and one in Uruguay. Few studies were conducted in Asia, with most data available on Japan and China[3]. In general, a trend of increasing prevalence has been correlated with increase in latitude.

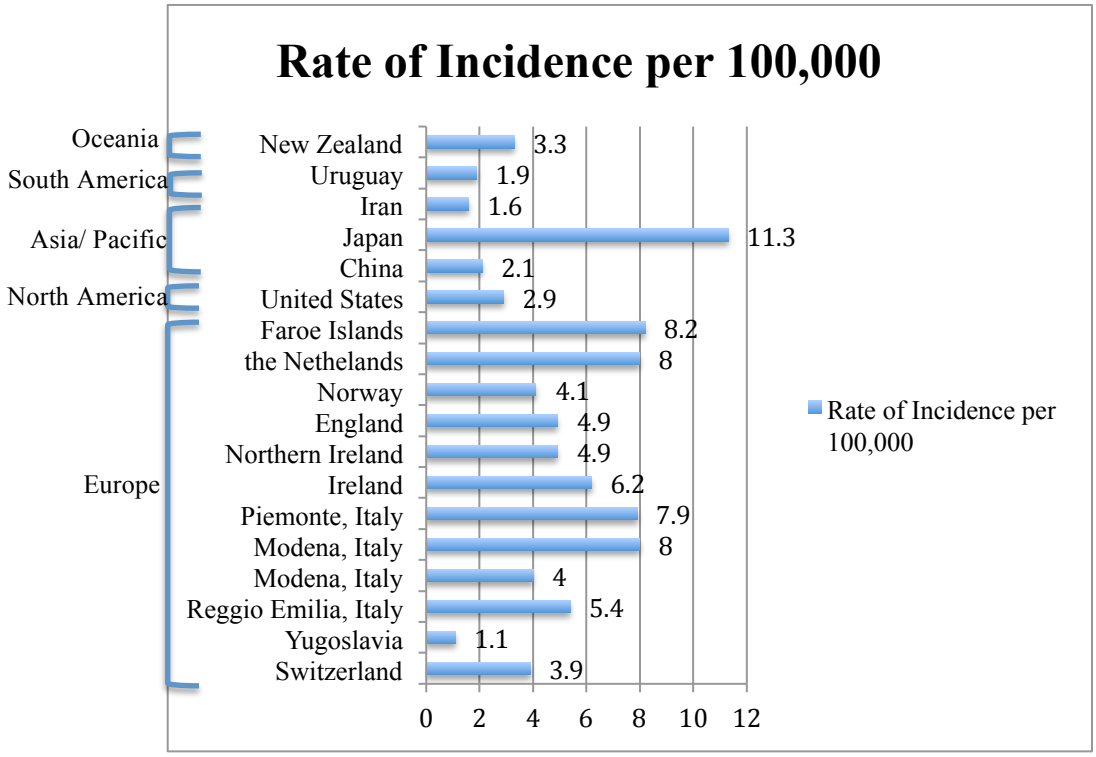

Figure 1: Rate of Incidence of ALS per 100,000 across different regions of the word[3]. Incidence was plotted as a function of the median age of the population due to increased incidence with advancing age.

Based on limited available data, ALS incidence is ten fold across countries when similar diagnostic criteria are applied, despite the genetic, environmental, socioeconomic and other differences when similar diagnostic criteria are applied. The highest rate of incidence in Japan is at 11.3 per 100,000. Italy also has one of the highest rates of incidence at 8.0 per 100,000 people. Uruguay, the only country represented in South America, has one of the lowest rates of incidence at 1.9 per

100,000 people[3,12]. Within Japan, the increased rate of incidence has been suggested to be due to a cyanobacteria that is found in the seeds of the cycad Cycas Micronesica[13]. The reason for changes in rates of incidence can be attributed to variability and inclusivity of diagnostics, the population sampled, accuracy of registries, and environmental factors.

In order to help reveal candidates for further studies of disease etiology and disease progression as well as to further understand racial and ethnic variations a study conducted in the United States by Kaiser Permanente looked at almost 6000 cases in eight metropolitan areas. The age-adjusted annual incidence rate for Caucasians was 1.79 per 100,000 person-years, 0.80 per 100,000 person-years for African American/blacks, 0.76 per 100,000 person-years for Asians, and 0.57 per 100,000 person years for Hispanics based on El Escorial diagnostic criteria[14]. As population-based data are otherwise not present for some of these groups of people, these data give insight on potential genetic, social, and environmental contributions to incidence.

A study conducted in Cuba of mortality from ALS, though not strictly diagnosed by El Escorial diagnostic criteria due to lack of access to neurological services in rural areas, showed that mortality from ALS appears to increase with age[15]. The study also showed increased mortality from ALS from those of Spanish origin over those with African background, indicating those from European decent may have a higher rate of incidence.

Population-based studies thus far have been limited by the diversity of the populations sampled. Currently, data are lacking from Latin America, Asia/ Pacific,

Oceania, and Africa. Most of the currently available studies have been conducted throughout Europe and have shown a relatively even rate of incidence across the region, with small variations in specific populations (Figure 1). Though there is increasing knowledge on ALS epidemiology, additional large-scale studies using standardized El Escorial Diagnostic criteria are warranted to further understand the disease.

LIMITATIONS WITH POPULATION STUDIES

One of the first limitations that need to be taken into consideration is the definitional components of ALS for clinical and observational studies, such as looking at a general population or a population sub-group and whether or not to look at incidence, prevalence and lifetime risk of ALS as being constant.

One of the biggest challenges is to clearly define the inclusion criteria for certainty of ALS and the phenotypic variants that are a part of the spectrum. The World Federation of Neuroscience has determined the El Escorial as the accepted diagnostic criteria, but older and smaller studies often don't follow this[15]. This requires using generally acceptable data collection instruments and programs, including questionnaires, which allows for more accurate comparison across studies as well as for data to be combined across studies. This can be more difficult to achieve in countries that do not have uniform access to healthcare and the neurological services required for diagnosis. These global variations offer an explanation as to why population studies are so limited because insufficient data are available to determine true rates of incidence of ALS.

Another limiting factor is the research done to determine environmental risk and taking into account background population genetics and sub-groups within select populations. Environmental risk factors include exercise, soccer, smoking, heavy metals, agricultural toxins, arduous manual labor, armed services, geographical clustering, and consumption of cyanotoxins[1,2,5,11,16]. The lack of demographic and phenotypic comparisons across geographical populations is also restrictive.

GENETIC FACTORS OF ALS

ALS is a rare disease, and the genetic factors leading to phenotypic presentation are also not identified in many studies. So far at least eight different genetic mutations have been associated with the development of ALS[1,7]. Cu/Zn Superoxide dismutase (SOD1) was the first gene identified and is a major cytoplasmic antioxidant enzyme that metabolizes superoxide anion and converts them to oxygen and hydrogen peroxide. Mutations in SOD1 represent the most common genetic abnormality, with more than 170 mutations reported[17]. Mutations in SOD1 influence different toxic stresses that lead to mitochondrial. Also SOD1 mutations could be involved in disruption of axonal transport and may result in the upper motor neurons (UMNs) and lower motor neurons (LMNs) being vulnerable to the disease progression[18]. The SOD1 gene has been the focus for the current, most widely used, disease model as well as treatment.

Other major genes implicated in the pathogenesis ALS are TAR DNA-binding protein (TARDBP), fused in sarcoma (FUS), optineurin (OPTN), valosin-

containing protein (VCP), ubiquillin 2 (UBQLN2), C9ORF72, sequestome 1 (SQSTM1), and profilin 1 (PFN1)[19]. The hexanucleotide repeat expansion in C9ORF72 accounts for about 40% of fALS cases. Research studying C9ORF72 has been hampered by the difficulty in producing a motor neuron disease model by these mutations. This is why ALS models are often based on SOD1 mutations now.

CURRENT DISEASE MODEL AND TREATMENT

The most frequent variant changes the alanine at position four to a valine, which results in an aggressive progression of ALS symptoms. Therefore, the first transgenic mouse model incorporated this A4V mutation. But, the mice did not present the ALS disease phenotype and so a different transgenic mouse line was created. The new line contained a mutation that changes the glycine at position 93 to an alanine[20]. The $SOD1^{G93A}$ mouse model has been determined to be a model that has helped to better understand where and when pathogenesis begins because it presents an observable disease phenotype.

To further characterize the pathological events as well as test possible drug treatments a transgenic mouse model of ALS was created in 1993, just one year after the identification of mutations in SOD1[21]. Many mutant transgenic mouse models have now been created that focus on specific mutations within the gene. However, no drug or treatment has been able to extend life by more than a month[22]. It was found that 2-7% of all familial cases are linked with a mutation in SOD1.

The SOD1 mouse model has thus far best captured the human condition and has furthered the understanding of disease pathogenesis. The only FDA approved disease-modifying drug for ALS is riluzole, a benzothiazole, which has extended life by three months in humans and two weeks in mice[23]. This drug is proposed to work by stabilizing voltage dependent sodium channels and increasing glutamate uptake activity, but its mechanism is far more complicated[24].

Twenty years of testing in transgenic SOD1 mice has not led to a promising treatment, presumably because of deficiencies in the design of the animal model and also due to the complex nature of ALS[25]. This has led the Beckman lab study conditions that would accelerate the disease phenotype in mice[25]. Son and Elliot discovered the disease phenotype could be greatly accelerated in the SOD^{G93A} mouse model by co-expressing the human copper chaperone for SOD1 (hCCS), leading to a promising treatment that is further discussed in the following chapter[26].

Chapter 2: ALS Research Advancements in the Beckman Lab

INTRODUCTION

Lou Gehrig's Disease, or Amyotrophic lateral sclerosis (ALS), is a disease caused by the death of motor neurons controlling voluntary muscle movements[16]. Prevalent factors associated with inherited forms of familial ALS (fALS) are mutations in the Cu/Zn Superoxide Dismutase (SOD1) gene, which encodes cytosolic SOD1[27,28]. In fALS, cytotoxicity of motor neurons appears to result from a gain of toxic SOD1 function, rather than from a loss of dismutase activity[29].

The widely expressed SOD1 uses copper redox chemistry to scavenge the free radical *superoxide*, a toxic oxygen species generated biologically by a number of means, including inadvertently during normal mitochondrial respiration and by phagocytes as an oxygen-dependent killing mechanism[27] (Figure 2). While SOD1 normally protects the cell from the harmful effects of superoxide accumulation, mutations in SOD1 result in a toxic gain of function that is responsible for a 2-7% of cases of the disease in humans[30,31,27]. There are over 170 associated fALS SOD1 mutations; one such mutation changes the glycine at position 93 to an alanine in SOD1, which disrupts the delicate copper and zinc binding and leads to misfolding[30].

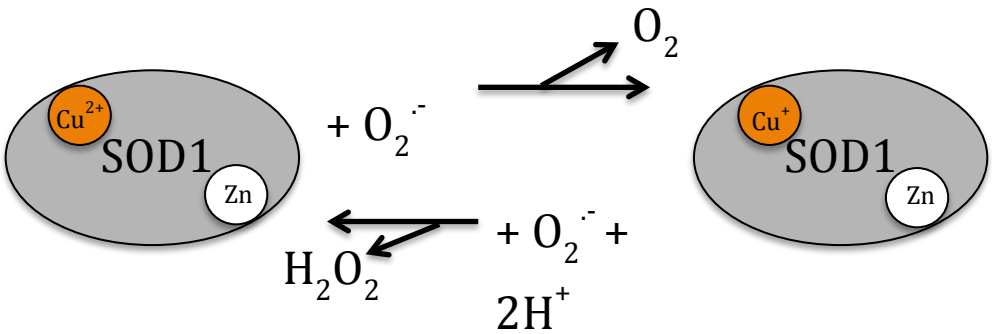

Figure 2: Reaction of SOD1 with radical oxygen species. Zinc acts as a stabilizing factor while copper participates in the reaction converting radical oxygen species to hydrogen peroxide.

The copper chaperone for SOD1 (CCS) is a 29kDa protein that helps convert immature monomeric SOD1 to fully mature Cu/Zn SOD1 by directly inserting a copper atom into the metal binding site and catalyzing the formation of an intra-subunit disulfide bond (Figure 3)[32]. Both CCS and SOD1 are naturally co-expressed in humans. SOD1 and CCS are found in the same cells and SOD1 is far more abundant than CCS. CCS provides a catalytic role in the maturation of SOD1[33].

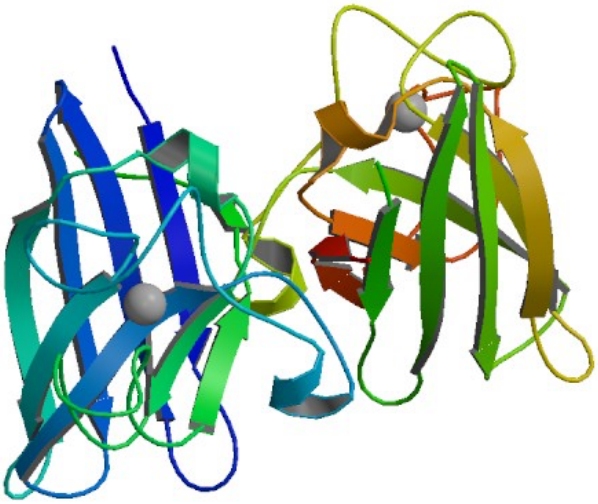

Figure 3: Crystal structure of the human copper chaperone for SOD1 (hCCS). PDB# 1D05

Previous work done in the Beckman lab using the SOD^{G93A} mouse model has shown that there are large amounts of SOD1 in the spinal cord and brain primed to receive copper from CCS to yield mature Cu/Zn SOD (SOD1). SOD1 lacking Cu^{2+} cannot catalyze the conversion of superoxide into hydrogen peroxide. Copper is a cofactor in essential catalysis and structurally for SOD1. When there are high amounts of copper-deficient SOD1, CCS will shuttle the copper away from its other important roles in the cell, such as in complex IV of the electron transport chain

(ETC), to form the mature Cu/Zn SOD[34]. Because of SOD1's high copper affinity it may serve as a copper sink reducing the copper pool available to other proteins[35]. This suggests why co-expressing with hCCS could impose large copper deficiencies in cytochrome *c* oxidase and ceruloplasmin.

So far there has been no treatment to significantly extend the life of high expressing SOD^{G93A} ALS model mice. The Beckman lab has demonstrated that the copper ligand CuATSM can slow the progression of ALS symptoms in the standard G93A model and and essentially stop the progression of ALS symptoms in the $SOD^{G93AxhCCS}$ mice. CuATSM has been shown to provide enough copper to CCS to drive all the partially folded SOD to the fully mature Cu,Zn SOD[36]. By better understanding the human CCS expression levels in transgenic mice we can test whether the CuATSM would be an effective treatment for human ALS.

The SOD^{G93A} mouse model has been well characterized, but important information about the G93AxhCCS line is lacking[21]. Namely, the relative abundance of SOD1 and hCCS protein levels are not well-characterized. Further study is needed to determine the ratios of CCS to SOD1 in the mouse model expressing human CCS. The expression levels of endogenous mouse CCS to human SOD1 is expected to be much lower than the ratio of hCCS to SOD in ALS patients[33]. Information on co-expressing hCCS with SOD1 will tell us whether the ratio of these proteins is similar to that found in human ALS patients. These results will help to evaluate whether effective treatments in the mouse model can be extended to humans.

Because all humans naturally express CCS and SOD1, we hypothesize that the $SOD1^{G93A}$xhCCA mouse more closely models the human ALS disease phenotype

than does the commonly-used G93A SOD1 mouse[21]. To test this hypothesis, I used quantitative western blots to assay the expression levels of SOD1 and CCS in both transgenic mouse tissue. In the work done for this thesis I examined the relative expression levels of hCCS and SOD1 in transgenic mouse tissue. My results will help further understand the $SOD1^{G93A}$xhCCS mouse model.

MATERIALS AND METHODS

Plasmids and Transformation: Human CCS cDNA was cloned into the pET3d vector and transformed into One Shot BL21(DE3)pLysS *Escherichia coli* (ThermoFisher) for protein expression. hCCS cDNA Nucleotide Sequence (NP_005116.1)

hCCS expression and purification. An overnight culture of transformed CCS BL21 *E. coli* was grown in Luria Broth containing ampicillin (100µg/mL) and chloramphenicol (34µg/mL) at 37°C shaking at 220rpm. This culture was diluted to an Optical Density (OD) reading at 600nm of 0.1 and then grown to an OD600 of 0.7 at 37°C, shaking at 220rpm for three hours. Expression of CCS was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1mM, after which incubation temperature was decreased to 18°C for 16 hours. Cells were harvested by centrifugation at 10,000xg at 4°C for 10 minutes, resuspended in 5mM 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) pH 7.0, and frozen overnight. Following freeze/thaw lysis, DNAase enzyme was added to a final concentration of 10µg/mL, incubated at 37°C for 20 minutes and centrifuged at 10,000xg at 4°C for 10 minutes. Residual DNA was removed by the addition of 0.05%

polyethylenimine (Sigma Aldrich) followed by additional centrifugation. The resulting supernatant was dialyzed against 4L of 5mM HEPES + 10mM 2-Mercaptoethanol (BME), pH 7.0 before purification via anion exchange chromatography. A 30 mL column of Poros 20 HQ (BioRad) medium was equilibrated with 5mM HEPES before introduction of the lysate. A gradient from 5 mM HEPES to 5 mM HEPES +1 M KCL was run with CCS eluting at 200 mM KCl. Protein concentration in collected fractions was determined by a microplate bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce). Purity of CCS was assessed to be greater than 90% by SDS-PAGE and western blotting.

SOD1 expression and purification. SOD1 was expressed in *E. coli* and purified as previously described[37].

Tissue Preparation. Mouse brain (primary motor cortex), spinal cord (lumbar or thoracic anterior grey column), and hindlimb muscle (gastrocnemius) were collected from SOD^{G93A}, $SOD^{G93AxCCS}$, and non-transgenic animals of varying ages. Mouse breeding and husbandry were previously described[25]. Biopsy punches 0.5 mm in diameter were taken from 1.0 mm-thick frozen tissue slices and weighed accurately to ±1 μg using Cahn 25 Automatic Electrobalance. Tissue punches were homogenized in buffer (50 mM Kpi, 20 μM EDTA, 0.1% Triton X-100) added to a final concentration of 15 μg wet tissue mass per microliter. Total protein concentration for each homogenate was determined using a microplate BCA protein assay (Thermo Scientific Pierce).

SDS-PAGE and immunoblotting. All electrophoretic gels were run using the Laemmli method on the MiniProtean system (Bio-Rad) according to the Bio-Rad SDS-PAGE manual for Tetra Cell[38]. All resolving gels were 12% acrylamide cast in 1.5mm molds. Samples were denatured in SDS loading buffer at 98°C for 8 minutes. Six micrograms total protein were loaded into each well and run in triplicate. Electrophoresis was carried out at 150V for 65 min.

For western blotting, samples were electrotransferred at 100 V for 100 minutes to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked overnight at 4°C with 5% fish gelatin (Sigma) in Tris-buffered Saline + 0.05% Tween-20 (TBST). Rabbit primary antibodies, SOD1 (prepared by Beckman lab), hCCS (Santa Cruz) and B-actin (Sigma), were applied in a 1:5,000 to 1:10,000 dilution in 1% gelatin in TBST for 2 h at room temperature. The membranes were then rinsed thoroughly with TBST and incubated for 30 min with 1:10,000 dilution of Li-Cor goat anti-rabbit secondary antibodies in TBST. The membrane was visualized using the Odyssey System (Li-Cor Biosciences).

Densitometric Analysis. Background-subtracted densities of bands were obtained using Odyssey software. ß-actin was used as a loading control in tissue samples. Normalization was performed by dividing the background-subtracted intensity of our protein bands of interest (PI_{raw}) by the density of ß-actin (NC_{raw}) in each lane relative to the lane with the greatest ß-actin signal (NC_{max}) (Eq 1 and 2).

Eq 1. $NC_{raw}/NC_{max} = NC_{rel}$

Eq 2. $PI_{raw}/NC_{rel} = PI_{normalized}$

RESULTS

hCCS Expression and Purification

Protein was expressed to a low bacterial density and at low temperatures to prevent aggregation and cells were grown to an OD600 of 0.7. The hCCS as described in methods was successfully expressed and purified. An uninduced, 5mL expression was grown along side the large scale expression as a negative control to ensure the media and IPTG were functioning properly and that the protein expressed is, in fact, CCS . Protein confirmation was made via SDS-PAGE as well as a western blot using CCS antibodies to confirm the purified protein is hCCS (Figure 4). Protein concentration was determined using a BCA assay and determined to be 1mg/mL (Figure 5, Table 3). I observed and purposefully had low expressing protein in order to prevent aggregation, as CCS is prone to do, especially when lacking copper[30].

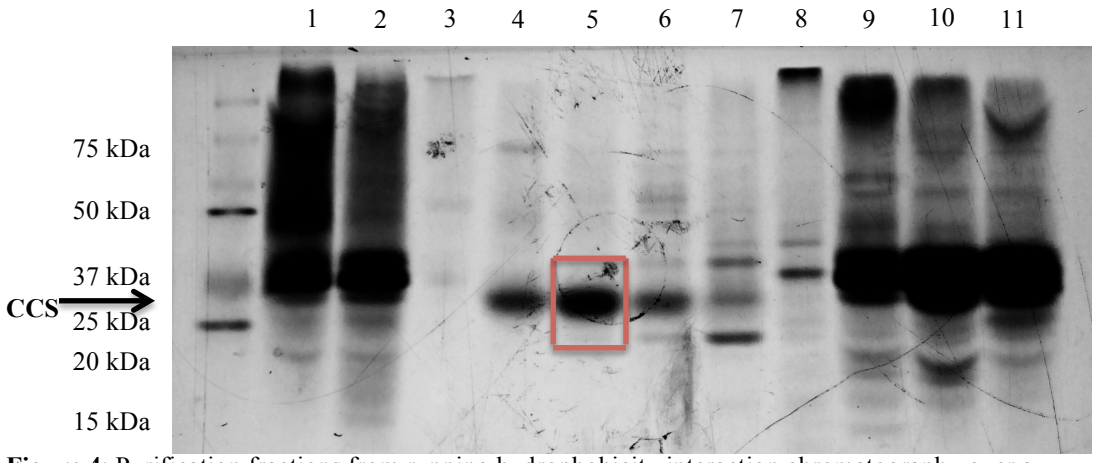

Figure 4: Purification fractions from running hydrophobicity interaction chromatography over a gradient from 5mM HEPES to 5mM HEPES + 1M KCl. The pure fraction, lane 5, eluted around 5mM HEPES + 100mM KCl.

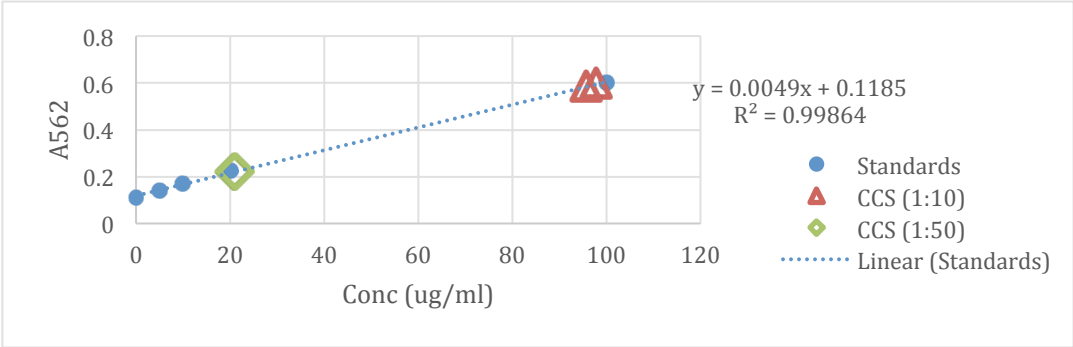

Figure 5: BCA of purified hCCS. Bovine SOD standard curve ranging from 10µg/mL bovSOD to 100µg/mL bovSOD. CCS samples were diluted both 1:10 and 1:50 to fit into the linear range of the curve.

Table 3: hCCS concentrations from BCA assay averaging the concentrations determined by both the 1:10 and 1:50 dilutions.

Samples (purified CCS)	**A562**			**Calculated concentration (ug/ml)**			**Dilution correction**		
Dilution	Rep1	Rep2	*Avg*	Rep1	Rep2	Avg	Rep1	Rep2	Avg
1:10	0.598	0.587	0.5925	97.86	95.61	96.73	978.57	956.12	967.34
1:50	0.22	0.222	0.221	20.71	21.12	20.92	1035.71	1056.12	1045.92

Validating antibody specificity

When selecting antibodies, I used the SOD1 antibody that our lab made that is a rabbit, polyclonal primary antibody. This antibody showed some cross reactivity between SOD1 and hCCS, possibly due to the considerable structural homology between the domain II and SOD1 itself. The CCS monoclonal antibody showed no cross reactivity for SOD1 (Figure 6).

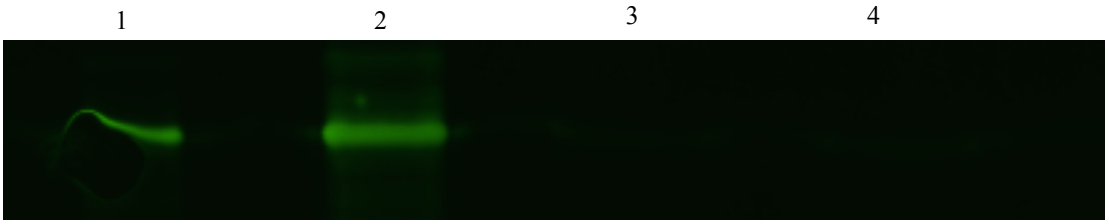

Figure 6: Characterization of reactivity of CCS antibody with hCCS and SOD1. Western blot of hCCS expressed in *E. Coli* (lanes 1,2) and SOD1 showing no cross reactivity between the CCS antibody and SOD1. The concentration of CCS was determined by cell count based on OD600=1 and 0.1(15µL per lane). The concentration of SOD is 5.0µM (lane 3) and 1.0µM (lane 4) (15µL per lane). Western was imaged using Li-Cor Technology.

ß-actin is a highly expressed protein found in both mouse and human tissues and was used as a loading standard to make sure that the same total amount of protein is being added and to be used for normalization for densitometry. Li-Cor secondary antibodies were required due to compatibility with the Odyssey instrument used for imaging. The infrared light excites the secondary antibodies due to an attached IR probe, causing them to fluoresce so that they may be visualized. A western blot was run using 6 day-old SOD^{G93A}xhCCS mouse tissue to test the sensitivity and specificity of each antibody used for future experiments and found that all showed specificity for their respective antigens (Figure 7). Standards for quantitation were included and ranged from 25 ng-100 ng (Figure 8)

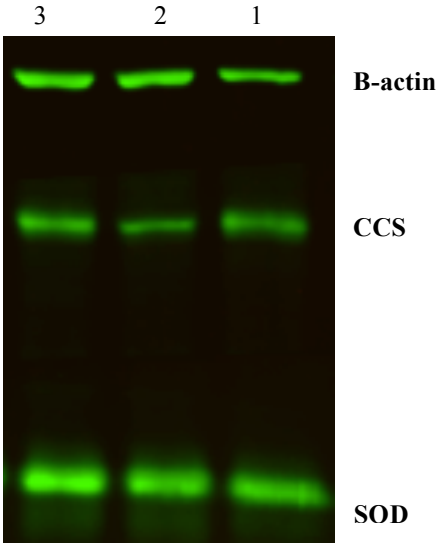

Figure 7: Evaluated antibodies for hCCS, SOD1 and actin to ensure antibody activity and confirm presence of CCS and SOD in the 6 day-old SODxhCCS mouse tissue.

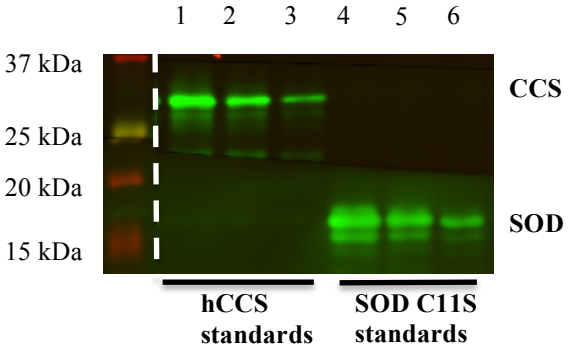

Figure 8: Confirmation of hCCS and SOD standards expressed in *E. coli* used for future quantization of protein in mouse tissues.) The concentration of hCCS (lanes 1-3) and SOD (lanes 4-6) standards was determined by BCA Assay with 100,50, and 25 nanograms of protein loaded, respectively

Expression differences between dorsal and ventral spinal cord

We were also interested in examining differences in expression levels between tissues. We found that there was no difference relative intensity in the location

between the dorsal and ventral regions of the spinal cord (Figure 9).

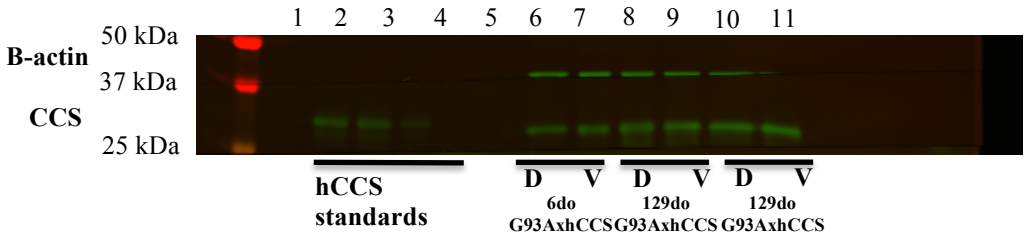

Figure 9. Comparison of relative quantities of hCCS and the dorsal vs ventral spinal cord of G93AxhCCS mice. hCCS expressed in *E.Coli* standards (lanes 2-4) The concentration of hCCS standards was determined by BCA Assay with 50, 25 and 10 nanograms of protein loaded, respectively. 6ug of total protein from each cord sample homogenate was loaded(lanes 6-11). Western was imaged using Li-Cor Technology.

Expression variation between tissues: brain and spinal cord

hCCS was more highly expressed in the motor cortex of brains or the lumbar region of the spinal cord. Looking at six day-old mice of the same genotype, there was greater expression of hCCS in spinal cords than in brains (Figure 10-A). Densitometry analysis on this western and found that hCCS was expressed 1.5 times greater in the spinal cord than in the brain while SOD levels remained relatively constant (Figure 10-B).

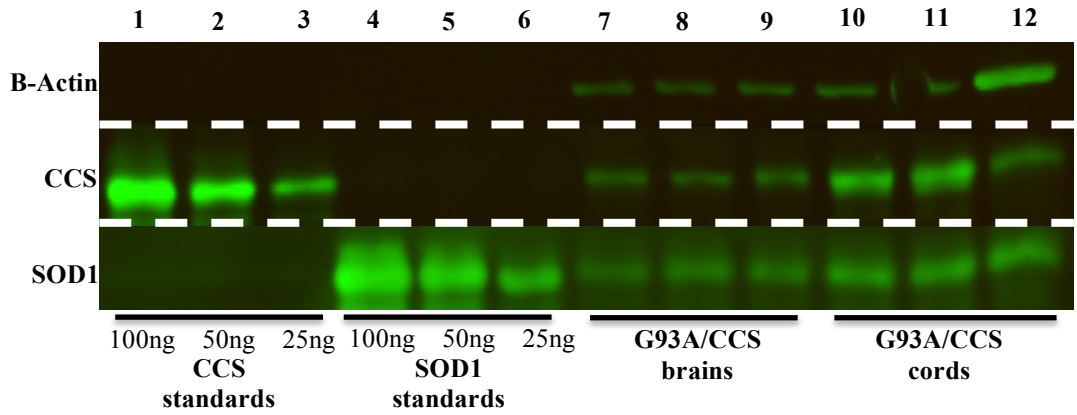

Figure 10– **(A)** Representative quantitative western blot. Recombinant proteins (human CCS and human C111S SOD1) grown in *E. coli* were used to construct standard curves for densitometry analysis (lanes 1-6). Four micrograms total protein per lane were loaded from biopsy punch homogenates of mouse primary motor cortex (lanes 7-9) and anterior grey column (lanes 10-12). Antibodies against B-Actin (~42kD, loading control), CCS (29kDa), and SOD1 (16kDa).

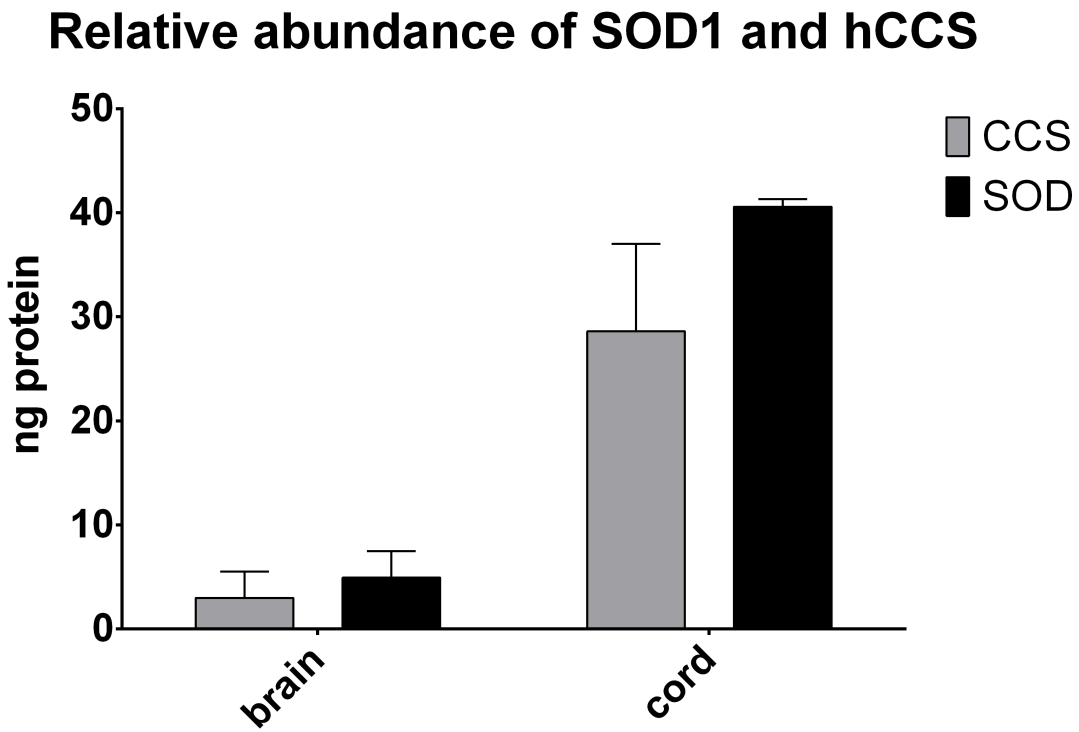

Figure10: – **(B)** Relative abundance of SOD1 and hCCS in the brain and spinal cord of $SOD1^{G93AxhCCS}$ six day old mice. Concentrations calculated by interpolation of standard curve. Error bars calculated using SEM.

DISCUSSION

In the SOD1^{G93A} mouse model there are 27 genomic copies of human SOD as well as the two endogenous mouse copies and there are only two copies of endogenous mouse CCS (mCCS). Even if there were a large increase in the expression of the mCCS, there would not be enough to fulfill its role as a copper chaperone for SOD1 and catalyze the maturation of SOD1. This leads to an accumulation of copper-deficient SOD1 resulting from the low amount of mCCS delivering copper to SOD1. By co-expressing with human CCS in the SOD1^{G93AxhCCS} model, it better resembles the balance of CCS and SOD1 found in humans and the potential response to the CuATSM treatment.

The ultimate purpose of this endeavor was to validate our mouse model by comparing the relative abundance of hCCS and SOD1 in the brain and spinal cord tissue in mice to that of human tissue. Though I wasn't able to confirm this, I was able to make meaningful strides towards reaching that goal. From my experiments I was able to further optimize the protocol for expressing and purifying hCCS in *E. coli* and develop a protocol for running western blots examining abundance of hCCS and SOD1 as well as a way to quantify that data.

Looking at the western blot comparing relative expression of hCCS and SOD in the dorsal vs ventral spinal cord, there qualitatively doesn't appear to be any notable difference in the relative expression, indicating that both could be used for future comparison with human tissue.

These results indicate interesting trends and correlations between age, tissue type and disease progression. Looking at Figure 10 I saw increased abundance of hCCS. Greater gene copy number, and therefore expression, of mutant SOD1 results in earlier onset of the disease as well as more severe disease symptoms and rapid disease progression[36]. It is clear that increased expression of hCCS in mice even further accelerates disease progression. In humans, SOD1 and CCS are present together in cells that degenerate in ALS[33]. By coexpressing hCCS along with mutant SOD1 in our mouse model, we think that we have more accurately modeled the human system. Further studies need to be done in order to confirm this.

Future experiments could look to confirm that the brain and spinal cords are the best tissues to look at for making a comparison between humans and our mouse model. We could also look to see how SOD1 metallation differs between comparable human and mouse tissue to determine if hCCS has the same catalytic ability, which would correspond with relative abundance proportional to the amount of SOD1 in the tissue. An important distinction that also needs to be made is determining the expression levels of endogenous mouse CCS compared to the over-expressed human CCS in the tissues we are examining.

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