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Toxicity and solubility of mutant superoxide dismutase 1 in amyotrophic lateral sclerosis

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Neuroscience

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An abstract of

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Abstract

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By Terrell Eileen Brotherton

Mutations in the gene encoding superoxide dismutase 1 (SOD1) account for approximately 20% of cases of familial amyotrophic lateral sclerosis. It is not known how the mutant protein causes disease, nor why only a subset of cell types (i.e. motor neurons) are targeted. The propensity of mutant SOD1 to form aggregates selectively in pathologically affected tissue has implicated these poorly soluble protein aggregates as being instrumental to the disease process. However, because aggregates are composed of misfolded SOD1, it has been difficult to distinguish the effects of aggregates and their misfolded, soluble precursors. Instead of being cytotoxic, aggregates may exist as a protective compensatory mechanism to sequester misfolded soluble protein, thereby decreasing aberrant cytosolic interactions. Here, I present data supporting the hypothesis that misfolded soluble, not insoluble aggregated, SOD1 is cytotoxic.

In this dissertation, I investigate several aspects of mutant SOD1 as it relates to cellular toxicity and the pathogenesis of ALS. I first present clinical data on a novel SOD1 mutation in familial ALS patients. This mutation, $SOD1^{C6S}$, presents with a mixed phenotype that includes an unusual characteristic of reduced disease penetrance. I then characterize an antibody, the C4F6 antibody that specifically marks "toxic" mutant SOD1. This antibody distinguishes pathologically affected and healthy tissue both in humans and in rodent disease models. Interestingly, C4F6 preferentially recognizes easily soluble, not insoluble, mutant SOD1 suggesting that toxic mutant SOD1 is soluble.

Finally, I investigate the relative solubility and cytotoxicity of separate SOD1 mutants. Through manipulating protein quality control pathways, the effects of aggregates and misfolded, soluble aggregate precursors are distinguished. I demonstrate that increased solubility is associated with increased toxicity, and furthermore, that decreasing the presence of misfolded, soluble SOD1 ameliorates this toxicity regardless of the presence of insoluble SOD1. Additionally, I demonstrate that increasing mutant SOD1 solubility exacerbates mutant SOD1-associated cytotoxicity. These findings implicate easily soluble, misfolded SOD1 as being toxic to the cell, and support the hypothesis that reducing solubility of mutant SOD1 proteins through aggregation may occur as a self-protective response in the cell.

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Chapter 1:

Introduction and Background

Introduction to Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, is an adult onset neurodegenerative disease that selectively targets motor neurons. ALS manifests as muscle weakness, rapidly progresses to paralysis and causes eventual death due to respiratory failure. The incidence of ALS is estimated at 1-2 persons per 100,000, with an average time to death after diagnosis of 3 to 5 years (Yoshida et al., 1986; Williams and Windebank, 1991; Millul et al., 2005a; Czaplinski et al., 2006a; Czaplinski et al., 2006b). The rapidity with which paralysis and death follow disease onset emphasizes the vital importance of treating this disorder. To date, no truly effective therapy for ALS has been found – it is fatal in all cases.

In ten percent of patients there is a family history of the disease, typically in a first degree relative (Siddique et al., 1991). An estimated 20% of these familial ALS (fALS) cases are caused by mutations in the gene coding for Cu/Zn superoxide dismutase 1 (SOD1) (Battistini et al., 2005; Logroscino et al., 2008a), and transgenic animals expressing mutant human SOD1 develop an ALS-like disease. SOD1 is a ubiquitous cytosolic enzyme responsible for scavenging harmful free radicals in the cell (Longo et al., 1996), and thus the initial hypothesis was that, in patients carrying SOD1 mutations, ALS is caused by a loss of SOD1 function leading to oxidative stress and cell death. Indeed, human ALS patients show increased protein carbonyl levels in both brain (Ferrante et al., 1997) and spinal cord (Shaw et al., 1995), and peroxynitrate mediated oxidative damage has also been demonstrated in patient tissues (Beal et al., 1997). However, the majority of SOD1 mutant proteins that are associated with disease do not show altered enzymatic activity (Valentine and Hart, 2003).

In addition, mutant SOD1 disease models typically are created in animals or cells that possess endogenous SOD1 enzymatic activity. It is now commonly accepted that mutant SOD1 proteins cause disease by attaining a property that is toxic to the motor neuron (Valentine and Hart, 2003). The nature of this toxic gain of function has remained elusive – there are over 155 ALS-associated SOD1 mutations spread across much of the protein, so disruption of a specific region of the enzyme is unlikely to be responsible for disease. Interestingly, most pathogenic SOD1 mutations interrupt the proper folding of the SOD1 protein (Gaudette et al., 2000), and SOD1 protein aggregates are found specifically in pathologically affected tissue, both in fALS-SOD1 patients and in rodent SOD1 models of disease (Watanabe et al., 2001; Bergemalm et al., 2010; Forsberg et al., 2010). Therefore, SOD1 associated toxicity in ALS may be mediated through protein misfolding and/or aggregation.

This chapter is divided into two sections. The first section describes aggregation and mechanisms of protein quality control as they broadly apply to a spectrum of neurodegenerative diseases. The second section focuses specifically on the historical role of misfolded and aggregated SOD1 in ALS, and how current research is challenging the doctrine that SOD1 aggregation is detrimental to cell viability.

1.1: Aggregation across neurodegenerative diseases

Protein aggregation is the unifying characteristic across a number of neurodegenerative disorders (Sherman and Goldberg, 2001). These disorders include

Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, and the prion diseases. The prevalence of protein aggregates specifically in pathologically affected tissue suggests that these inclusions are associated with the disease process. It is not clear, however, whether aggregates play a protective role, are themselves the source of toxic neuronal insults, or are innocent bystanders in neuronal pathology (Stefani and Dobson, 2003; Bucciantini et al., 2004; Calloni et al., 2005; Baglioni et al., 2006; Hegde and Upadhya, 2007).

Protein aggregates are generally defined as multimeric complexes of abnormally folded proteins exhibiting decreased solubility (Kopito, 2000). These complexes can exist in either an ordered (*e.g.* β-pleated sheet) or amorphous structure. Moreover, aggregates are commonly accepted to exist as established structures that are not degraded through traditional proteolytic processes. Proteins may assume abnormally folded conformations through initial errors in translational processing, genetic mutations, or by the influence of an external stressor. Additionally, natively folded proteins are known to exist in a dynamic state, frequently passing through transient, partially unfolded intermediate conformations, which can further increase the prevalence of non-natively folded proteins (Wolfe and Cyr, 2011). As cells age or are exposed to external stressors, they exhibit an increased presence of misfolded proteins, which may ultimately accumulate as protein aggregates.

1.1.2: Protein misfolding, degradation, and quality control

Protein quality control mechanisms exist within the cell to screen for, and subsequently repair or degrade, improperly folded proteins. Molecular chaperone proteins are one such method of quality control; chaperones assist in nascent protein folding, recognition and subsequent unfolding and re-folding of misfolded proteins, and the targeting of misfolded proteins for degradation (as reviewed in (Hartl et al., 2011)). Two of the most commonly utilized pathways for protein degradation include the ubiquitin-proteasome pathway (UPP) and autophagy.

The ubiquitin-proteasome pathway plays a crucial role in proteolytic degradation. The core structural component of this pathway, the 26S proteasome, is a protein complex assembled from two basic subcomplexes, the 20S proteasome barrel and two 19S proteasome caps, one at each end of the barrel (Figure 1.1) (as reviewed in (Bedford et al., 2010; Rogers et al., 2010)). The 20S proteasome gets its characteristic barrel shape from 4 stacked rings, each composed of 7 polypeptide subunits. The two outer rings are designated α subunits, and the two inner rings, β subunits. The β subunits mediate proteolytic activity; there are three β subtypes (β1, β2, and β5) and each is responsible for cleaving peptide bonds after specific amino acid subtypes (Heinemeyer et al., 1997). β1 cleaves after acidic amino acid residues (post-glutamyl peptide hydrolase/caspase-like activity), β2 cleaves after basic residues (trypsin-like activity), and β5 cleaves after hydrophobic residues (chymotrypsin-like activity) (Glickman and Ciechanover, 2002). As proteolytic activity is mediated by β subunits, and the β rings are located in the interior of the proteasome catalytic core, the

substrate must first bypass the external α ring in order to be degraded. The opening in the α ring is relatively narrow (only 13 Å), essentially allowing the α ring to act as a "gate" for protein entry into the proteasome (Lupas et al., 1995; Wenzel and Baumeister, 1995). In order to pass through this "gate", the protein substrate must first be unfolded so that it can be threaded through the narrow α ring opening (Navon and Goldberg, 2001). Substrate recognition and unfolding are mediated by the 19S cap. Recognition is based upon the attachment of a minimum of 4 ubiquitin molecules to the substrate (and thus the designation "ubiquitin-proteasome pathway/system") (Thrower et al., 2000).

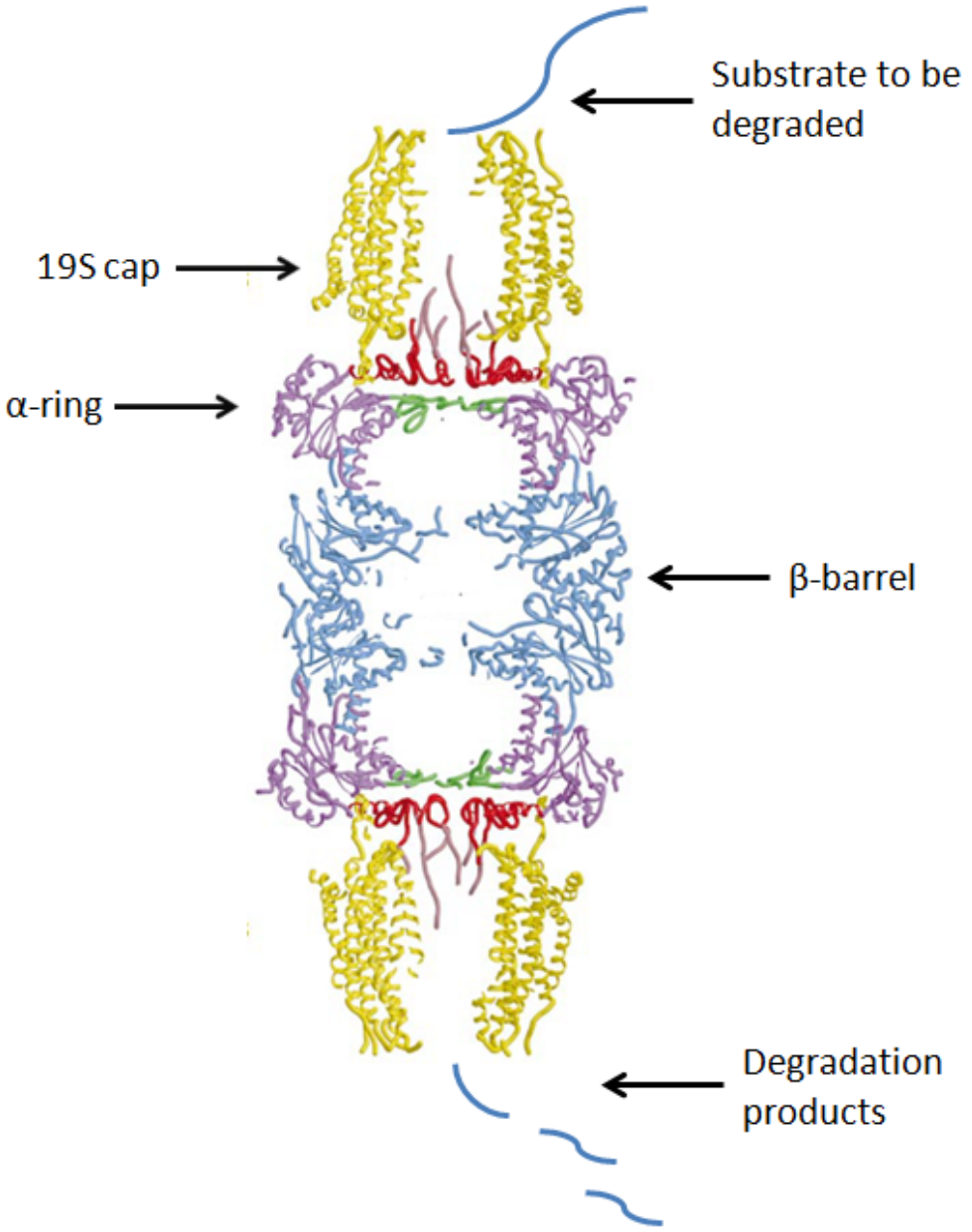

Figure 1.1. The 26S proteasome complex degrades misfolded and aberrant proteins. The 19S cap helps unfold and thread misfolded protein into the 20S proteasome barrel. The substrate is threaded through the narrow opening in the α "gate", and is then degraded by proteolytic activity in the β barrel catalytic chamber. Image is modified and reproduced with permission from Nature Publishing Group: Whitby et al, Nature 2000 (Whitby et al., 2000).

Substrate tagging refers to the process of tagging misfolded or otherwise aberrant proteins with ubiquitin in order to target them to the proteasome for degradation. These proteins are thought to be recognized by molecular chaperones as being ubiquitin substrates due to their abnormal exposure of hydrophobic residues (a characteristic of misfolded proteins) (Shimura et al., 2004; Urushitani et al., 2004; Husnjak and Dikic, 2012). Ubiquitination of these misfolded proteins is a highly specific process, involving three separate enzyme families, known as E1, E2, and E3 (Figure 1.2) (as reviewed in (Hershko and Ciechanover, 1992, 1998)). The function of E1 is to activate ubiquitin through an ATP-dependent reaction; E1 is the least selective of the enzymes in the ubiquitin-substrate linking pathway. E2, designated the ubiquitin-conjugating enzyme, becomes linked to ubiquitin in the second step of this process and is more selective than E1; there are estimated to be between 20-30 E2 enzymes in mammals. The most selective of the enzymes in this pathway is E3, the ubiquitin-protein ligase/substrate recognition ligase; there are estimated to be several hundred E3 proteins. E3 interacts with both the substrate to be degraded (or the molecular chaperone marking it as misfolded) and E2 in order to transfer ubiquitin from E2 to the substrate. The tagged substrate is then shuttled to the proteasome, where it is unfolded, and degraded. Importantly, all three enzymes are necessary for ubiquitination of the substrate. If any steps in this pathway are missing or disrupted, a build-up of misfolded proteins in the cell will result.

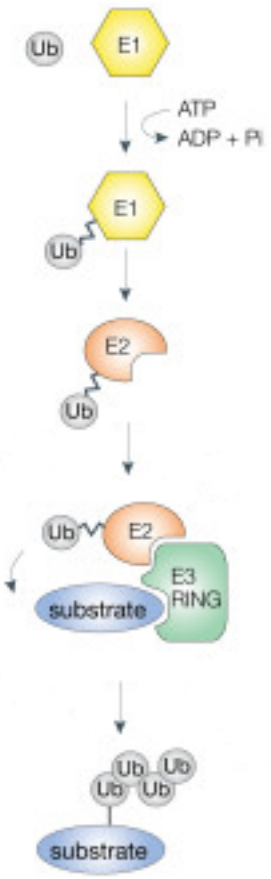

Figure 1.2. Ubiquitination of substrates to be degraded by the 26S proteasome. Ubiquitination begins with a ubiquitin-activating reaction. In this reaction, ubiquitin is activated and transferred to E1 in an ATP-dependent manner. Subsequently, ubiquitin is conjugated to the more selective ubiquitin-conjugating enzyme, E2. Finally, ubiquitin is transferred from E2 to the substrate to be modified by the highly selective E3 ubiquitin ligase. Image is modified and reproduced from Woelk et al, Cell Division 2007 under the terms of a Creative Commons license (Woelk et al., 2007).

Autophagic proteolysis represents another method of protein degradation. As this dissertation does not include autophagy studies, this method of degradation will be described only briefly. Autophagic substrates tend to be both bulkier and longer-lived than proteins degraded through the UPP (Ciechanover et al., 2000). Indeed, autophagy can degrade entire organelles, including proteasome sub-complexes (Cuervo et al., 1995). Autophagy, which takes its name from "self-eating", describes the process of lysosomal degradation. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Unless otherwise specified, autophagy will refer to macroautophagy. In this process, a double layered membrane known as the phagophore engulfs part of the cytoplasm (as reviewed in (Mizushima, 2007)). The phagophore eventually fuses with itself, sequestering its contents from the cytosol and becoming the autophagosome (Figure 1.3). The autophagosome is then transported to, and subsequently fuses with, the lysosome, allowing its cargo to be destroyed by lysosomal hydrolases (Berg et al., 1998; Stromhaug et al., 1998; Suzuki and Ohsumi, 2007).

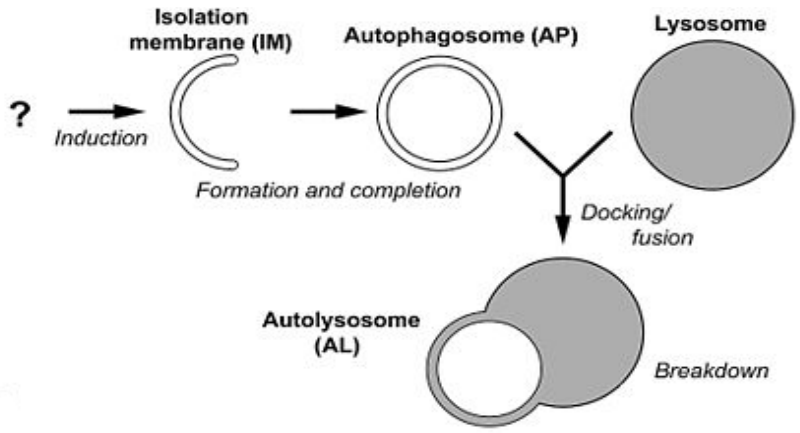

Figure 1.3. Autophagy pathway. The substrate to be degraded is initially engulfed in the cytoplasm by the double membraned phagophore. The phagophore eventually fuses with itself, sequestering the substrate within the newly formed autophagosome. The autophagosome then fuses with the lysosome, creating the autolysosome, and the substrate is degraded by lysosomal hydrolases. Image is reproduced with modification from Juhasz et al, PLoS Biology 2006, under the terms of a Creative Commons license (Juhasz and Neufeld, 2006).

Both autophagy and the UPP utilize molecular chaperones when targeting proteins for degradation, particularly chaperones in the heat shock protein (HSP) family. Named for their propensity to be up-regulated in response to heat shock, HSPs accumulate in times of cell stress and recognize unfolded or improperly folded proteins by their abnormal exposure of hydrophobic residues (hydrophobic residues are typically buried within the interior core of natively folded, soluble proteins) (Richarme and Kohiyama, 1993). Because HSPs recognize misfolded or unfolded proteins by their exposed hydrophobic residues, not by the presence of specific linear or conformational epitopes, these chaperones provide a general mechanism to recognize and rid a wide variety of improperly folded proteins from many different cell types. Heat shock proteins are either constitutively expressed or induced in response to a stressor. Their functions include *de novo* protein folding, re-folding of denatured or misfolded proteins, protein trafficking, and facilitation of proteotylic degradation by ubiquitin conjugation (as reviewed in (Morimoto, 1998, 2012) (Voisine et al., 2010)).

When protein refolding and degradation pathways become saturated and can no longer cope with the burden of improperly folded proteins, these abnormal proteins instead accumulate into aggregates. Past research has focused heavily on protein aggregates being inherently toxic to cells, but this line of inquiry has largely been based on the spatial correlation between protein inclusions and pathologically affected tissues, not empirical evidence of aggregate toxicity. None-the-less, the potential mechanisms of aggregate-induced cytotoxicity are worth considering. Aggregates could sequester proteins vital to cellular processes (Durham et al., 1997; Bruijn et al., 1998), depleting the cell's endogenous

supply of chaperone proteins and thereby decreasing its ability to repair misfolded or damaged proteins (Bruening et al., 1999; Shinder et al., 2001). The mere presence of protein aggregates induces stress on cellular organelles (*e.g.* the endoplasmic reticulum) and may impair the proteasome's degradation capabilities (Bence et al., 2001; Bence et al., 2005; Oh et al., 2008). Additionally, aggregates can impair protein transport by "choking" or blocking the transport machinery (Mandelkow et al., 2003; Lee et al., 2004; Duncan and Goldstein, 2006).

Elimination of aggregation-prone species has consistently been shown to slow pathology across neurodegenerative models (Yamamoto et al., 2000; Regulier et al., 2003; Zu et al., 2004; Lin et al., 2009). However, it is not clear if this "rescue" effect on viability is due to the elimination of fibrillar aggregates or to the elimination of their misfolded soluble precursors. Indeed, the question of toxicity in aggregates as opposed to their soluble, misfolded precursors, the "protoaggregates," has frustrated researchers for years. In this dissertation, the term "protoaggregate" does not distinguish between easily soluble misfolded, monomeric protein and easily soluble, oligomeric protein. Because aggregate formation necessarily depends upon the transient presence of protoaggregates, divorcing the effects of insoluble, aggregated and soluble, misfolded proteins has proven complex.

In recent years, investigators have devised creative experimental paradigms to separate the effects on cytotoxicity of aggregates versus soluble protoaggregates. Remarkably, these studies have implicated soluble protoaggregates, not insoluble aggregates, as being essential to toxicity. In an elegant study in the Alzheimer's field, slowing the aging

process in $A\beta_{1-42}$ expressing *C. elegans* by manipulating the insulin growth factor pathway increased *C. elegans* viability *without* decreasing $A\beta_{1-42}$ aggregation levels. Notably, levels of soluble oligomeric Aβ correlated directly with toxicity in this study (Cohen et al., 2006), supporting the hypothesis that soluble oligomeric, not insoluble aggregated, Aβ was toxic. Additionally, two separate studies have shown that pharmacologically increasing the size of mutant huntingtin and synuclein aggregates increases cell viability (Bodner et al., 2006; Outeiro et al., 2007). It is of importance to note that in these experiments levels of the diffuse cytoplasmic protein decreased as aggregate size increased, supporting the assertion that soluble, misfolded protein was indeed being sequestered into aggregates. Therefore, as the levels of soluble, protoaggregates decreased, cytotoxicity also decreased, and this remarkable rescue in viability was observed despite the increased presence of insoluble aggregates. These experiments strongly implicate soluble protoaggregates, and not the classically defined insoluble aggregates, in cytotoxicity.

1.2: Amyotrophic lateral sclerosis and superoxide dismutase 1

This dissertation focuses on the relationship between SOD1 aggregation and ALS. As described in the beginning of this chapter, 20% of patients with familial ALS possess mutations in SOD1. Furthermore, these mutated forms of the protein generally do not show a decrease in enzymatic activity (Valentine and Hart, 2003), supporting the hypothesis that SOD1 acts through a toxic gain of function. The nature of this gain of function is not clear; however, as aggregated SOD1 is found specifically in affected cell types, past theories have implicated protein aggregation as mediating ALS pathology.

1.2.1: Protein Aggregation in ALS

Protein aggregation in affected tissue is characteristic of both sporadic and familial ALS (Shibata et al., 1994; Shibata et al., 1996a; Shibata et al., 1996b). These aggregates encompass skein-like inclusions, round hyaline inclusions, Lewy body-like hyaline inclusions, and Bunina bodies. Bunina bodies are small, eosinophilic cytoplasmic inclusions that are considered to be a unique hallmark of ALS (Figure 1.4A). The composition of Bunina bodies has eluded scientists for years, with the only definitively identified proteins in these inclusions being cystatin C and transferrin (Okamoto et al., 1993; Mizuno et al., 2006). With the exception of Bunina bodies, many ALS associated inclusions stain positive for proteins implicated in protein turnover or RNA metabolism, and in fact, the majority of ALS tissue contain aggregates of the RNA binding protein, TAR DNA-Binding Protein 43 (TDP-43) (Arai et al., 2006; Neumann et al., 2006). The protein-quality control proteins ubiquitin, dorfin (an E3 ubiquitin ligase), and ubiquilin-2 are also frequently identified in inclusions (Migheli et al., 1994; Watanabe et al., 2001; Niwa et al., 2002; Deng et al., 2011). Whether sporadic ALS tissues contain SOD1 aggregates is a matter of debate, however it is clear that tissues from mutant SOD1-associated ALS patients do contain SOD1 aggregates (Figure 1.4B) (Rakhit et al., 2007; Liu et al., 2009; Bosco et al., 2010; Brotherton et al., 2012). These aggregates are present in motor neurons and occasionally in the surrounding astrocytes of SOD1-ALS patient tissue (Shibata et al., 1996a; Kato et al., 2000; Forsberg et al., 2011). SOD1 aggregates are also routinely identified in mutant SOD1-transgenic rodent models and in select *in vitro* cell systems (Bruijn et al., 1997; Johnston et al., 2000; Wang et al., 2002a;

Wang et al., 2002b; Aoki et al., 2005). Interestingly, the prevalence of high molecular weight and detergent-insoluble SOD1 is highest in pathologically affected tissue of SOD1 rodents, suggesting that these aggregates or their misfolded precursors, influence ALS pathogenesis (Johnston et al., 2000; Wang et al., 2002a; Wang et al., 2002b). In order to address the mechanisms through which SOD1 misfolding/aggregation contributes to cytotoxicity, it is first necessary to describe the protein's native, non-toxic, structure.

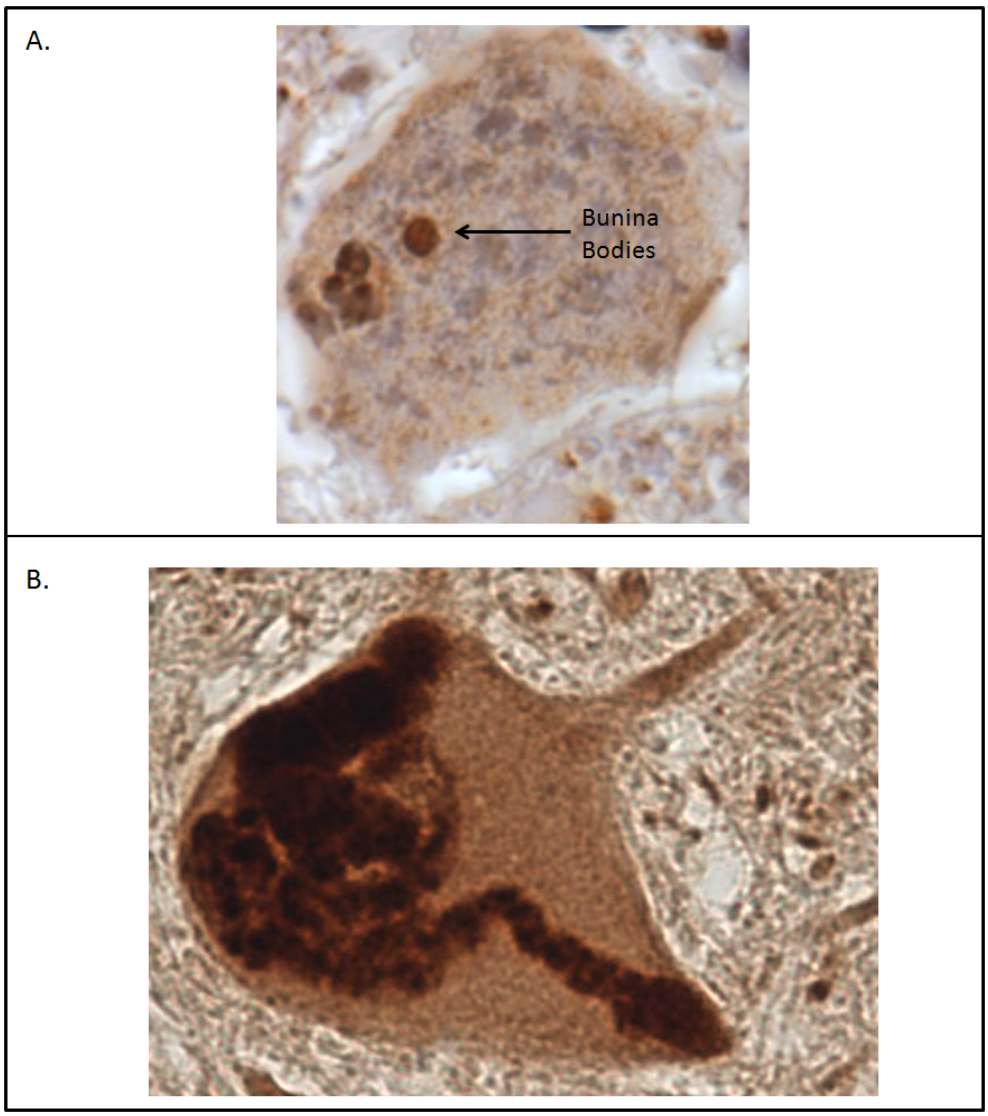

Figure 1.4. Protein aggregation in ALS patient tissue. A. Bunina bodies (arrow) are eosinophilic inclusions that are a specific marker of ALS pathology in affected spinal motor neurons. B. SOD1 aggregation in an $fALS^{A4V}$ spinal motor neuron. SOD1 aggregates are commonly seen in SOD1-fALS; note the skein-like nature of the inclusion. Image A is reproduced with modification from Momeni et al, BMC Neurol 2006 under the terms of a Creative Commons License (Momeni et al., 2006). Image B was obtained with the C4F6 antibody in our own laboratory.

1.2.2: Superoxide dismutase 1 structure

CuZn SOD1 protein monomers self-associate to form a remarkably stable homodimer (Arnesano et al., 2004). Notable structural features of each monomer include a highly conserved intrasubunit disulfide bond, a stabilizing zinc binding site, and a catalytic copper binding site (Tainer et al., 1982; Tainer et al., 1983). The relative importance of these features in maintaining protein conformation, as well as activity, has been a contentious topic in the ALS field, and it is not clear how alterations in each of these features contribute to SOD1 toxicity.

The SOD1 homodimer is composed of two identical 153 amino acid chains, each bound in an 8-stranded β-barrel formation (Figure 1.5) (Tainer et al., 1982). The dimer interface is maintained primarily through hydrophobic interactions (Tainer et al., 1982). Situated in each monomer's active site is a copper ion, which catalyzes the dismutase reaction for which SOD1 is named (Forman and Fridovich, 1973). In this dismutase reaction, SOD1 acts as an anti-oxidant, converting two molecules of superoxide anion ($O_2^{\cdot-}$) to O_2 and H_2O_2 (McCord and Fridovich, 1969; Forman and Fridovich, 1973). Copper is sequentially reduced (reaction I) and oxidized (reaction II) by $O_2^{\cdot-}$.

$$\text{SOD1-Cu(II)} + O_2^{\cdot-} \leftrightarrow \text{SOD1-Cu(I)} + O_2 \qquad \text{(reaction I)}$$

$$\text{SOD1-Cu(I)} + O_2^{\cdot-} + 2H^+ \leftrightarrow \text{SOD1-Cu(II)} + H_2O_2 \qquad \text{(reaction II)}$$

The zinc-binding (loop IV) and electrostatic (loop VII) loops define the channel leading to the active site (Tainer et al., 1982; Tainer et al., 1983). Loop IV is especially influential in maintaining the structure of the dimer interface, the intra-subunit disulfide bond, and the zinc-binding site (Hornberg et al., 2007). Loop VII helps guide superoxide radicals to the enzyme's active site (Getzoff et al., 1983). Therefore, the maintenance of both loop conformations is necessary to sustain a healthy dismutase equilibrium.

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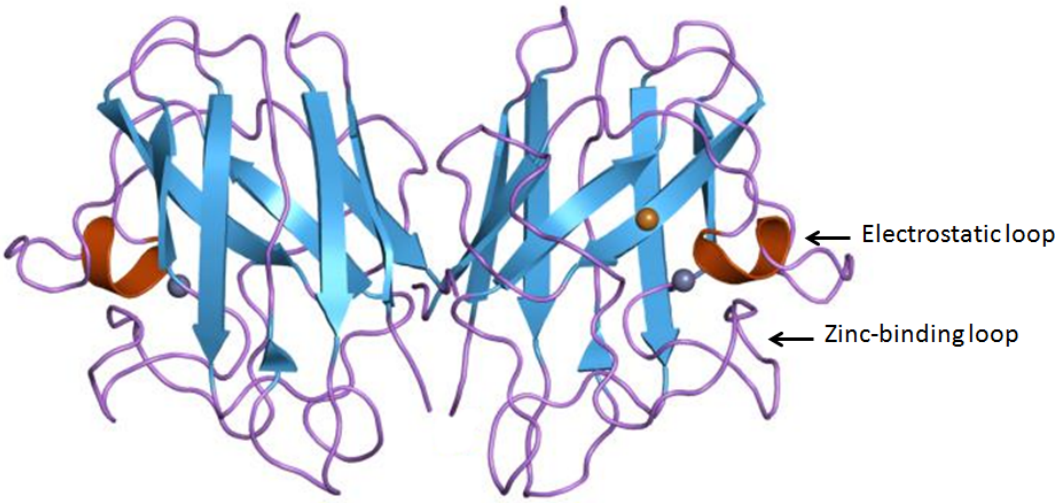

Figure 1.5. Structure of human superoxide dismutase 1. Note the homodimeric structure of the protein. Both the zinc-binding loop and electrostatic loop are necessary for maintaining the proper three-dimensional conformation of the protein, facilitating the dismutation reaction. Image reproduced with modification from Protein Data Bank structure 1spd (http://www.ebi.ac.uk/pdbe-srv/view/entry/1spd/summary) taken from the original paper by Deng et al, Science 1993, under the terms of a Creative Commons license.

1.2.3 Mechanisms of SOD1 misfolding

Natively folded SOD1 is a highly stable protein. Wild-type, fully metallated protein retains its activity in 8 M urea, 4% SDS, and at 80°C (Forman and Fridovich, 1973; Malinowski and Fridovich, 1979; Arnesano et al., 2004). The majority of ALS-associated SOD1 mutants, in contrast, show markedly reduced stability. SOD1 mutants are classified as "wild-type like" or "metal-binding region" mutants; wild-type like mutants retain proper copper coordination and full enzymatic activity, whereas metal-binding region mutants do not properly bind copper and/or zinc ions and tend to exhibit decreased activity (Hayward et al., 2002). Wild-type like mutants have a reduced melting point of 1-6°C relative to wild-type SOD1, whereas metal-binding region mutants exhibit a melting point 4-12°C below that of wild-type SOD1 (Rodriguez et al., 2002). If SOD1 stability and ALS pathogenesis are indeed linked, mutants with decreased stability would be predicted to be associated with shorter disease progression. This was found to be the case in a study investigating 29 ALS patients possessing 22 different mutations (Sato et al., 2005). However, other studies suggest that decreased stability does not correlate with disease progression (Prudencio et al., 2009; Vassall et al., 2011).

How over 155 mutations in a single protein can all lead to the same disease remains an enigma. The theory of protein misfolding as causative of ALS pathology provides an attractive, general solution to this problem. Further supporting misfolding as being a common "trigger" for pathogenesis, there are various mechanisms through which misfolding of the wild-type protein can be achieved, namely oxidative stress, metal depletion, and

reduction of the intrasubunit disulfide bond (as reviewed in (Valentine and Hart, 2003; Kabashi et al., 2007)). It is not clear which, if any, of these mechanisms provide the necessary insult, or trigger, to initiate SOD1 misfolding. These mechanisms may act in isolation, in concert with each other, or with some other as yet undiscovered stressor. While it is not known *how* misfolding of SOD1 is initiated within the cell, it is known that the injection of misfolded protein into the cell is toxic (Estevez et al., 1999), substantiating the idea of misfolded SOD1 as a contributing factor to neuronal damage.

Oxidative stress promotes protein misfolding in numerous proteins, including SOD1 (as reviewed in (Herczenik and Gebbink, 2008)). Mutant SOD1 showed structural changes, as detected through circular dichroism spectroscopy, when oxidized in the presence of ascorbic acid and $CuCl_2$ (Rakhit et al., 2002). When wild-type SOD1 was exposed to the same conditions of metal-catalyzed oxidation (MCO), the SOD1 dimer dissociated into aggregation-prone monomers, exposing previously shielded hydrophobic residues (Rakhit et al., 2004). Oxidation of $SOD1^{WT}$ with H_2O_2 was also shown to promote a novel association between $SOD1^{WT}$ and heat shock protein 70 (Hsp70) (Ezzi et al., 2007). Hsp70 commonly associates with mutant SOD1, promoting refolding or degradation of the misfolded, mutant protein (Shinder et al., 2001), and therefore it is postulated that oxidation of $SOD1^{WT}$ causes the protein to assume a misfolded conformation that is recognized and targeted by protein refolding machinery. Furthermore, hSOD1-transfected HEK293 cells exposed to oxidative stress form highly carbonylated SOD1 oligomers, suggesting oxidized SOD1 is prone to misfolding and subsequent aggregation (Urushitani et al., 2002).

Zinc depletion as a mechanism for SOD1 misfolding, and potential neuronal toxicity, has gained popularity in recent years. It is known that the delivery of either zinc-deficient mutant or zinc-deficient wild-type SOD1 to motor neuron cultures is sufficient to induce apoptosis (Estevez et al., 1999). Further implicating zinc depletion in pathogenesis, when both copper and zinc are in excess, neither mutant nor wild-type SOD1 were as toxic to these cultures (Estevez et al., 1999). It may be that an underlying cause for the toxicity of Zn-deficient SOD1 is its propensity to misfold. In the absence of zinc, the zinc-binding loop assumes a non-native conformation (Strange et al., 2003), altering the catalytic activity of SOD1. The zinc-deficient conformation alters exposure to the active site, which promotes catalysis of new substrates, such as peroxynitrite (Beckman et al., 1993; Crow et al., 1997). SOD1-mediated catalysis of peroxynitrite subsequently results in the nitration of tyrosine residues of other proteins (Beckman et al., 1992; Ischiropoulos et al., 1992). Interestingly Zn-deficient SOD1 preferentially catalyzes tyrosine nitration by peroxynitrite at two-fold the rate that zinc-replete SOD1 does (Crow et al., 1997), providing a plausible mechanism for zinc-deficient SOD1 mediated toxicity.

The specific conformational changes resulting from zinc depletion have recently been mapped. Loosening of both the zinc-binding and the electrostatic loops were noted in the absence of zinc (Rakhit et al., 2002). As described above, these loops line the active site channel, so disorder in these loops could lead to increased accessibility of the active site. A further change associated with the zinc-deficient homodimer was asymmetry of its subunits. The dimer subunits were found to be rotated 9º from their standard orientation in the absence of zinc, exposing a larger surface area of the dimer interface than is typically

accessible to the "outside" environment (Roberts et al., 2007). Zinc-deficient SOD1 therefore shows a looser structure, increased access to the catalytic copper, and an asymmetrical orientation allowing increased access to the dimer interface as compared to zinc-replete SOD1.

CuZn hSOD1 maintains an intrasubunit disulfide bond, an unusual feature for a cytoplasmic protein given the highly reducing environment of the cytosol. This disulfide linkage connects loop IV (Cys-57) and strand β8 (Cys-146) within each monomer. As described above, a loosening of loop IV results in disorganization of both the dimer interface and the zinc-binding site (Hornberg et al., 2007), allowing increased access to the catalytic site. This linkage is also instrumental in maintaining the relative conformation of residue Arg-143, a residue that has a direct role in regulating the catalytic site (Fisher et al., 1994; Banci et al., 2002; Banci et al., 2006). Therefore, reduction of the disulfide linkage could modify the conformation of SOD1 in at least three different ways – disruption of the dimer interface, disorganization of the zinc-binding site, and direct disorganization of the protein active site – and all of these modifications have potential repercussions on catalytic activity. Interestingly, fALS SOD1 mutants are more vulnerable to reduction of the intrasubunit disulfide than wild-type SOD1 (Tiwari and Hayward, 2003). Furthermore, pathologically affected tissues exhibit increased vulnerability to disulfide reduction in ALS rodent models; brain and spinal cord from $SOD1^{G93A}$ and $SOD1^{D90A}$ mice have higher steady-state levels of disulfide-reduced protein than non-CNS tissues (Jonsson et al., 2006). However, despite the increased presence of disulfide-reduced SOD1 in affected tissue, and the repercussions of breaking this bond on protein conformation, evidence suggests that the

disulfide-reduced form of SOD1 may not be cytotoxic. This is supported by the finding that hSOD1^{WT} transgenic mice actually possess higher levels of disulfide-reduced hSOD1 than do mutant hSOD1 models (Jonsson et al., 2006). Therefore, there may be structural alterations beyond reduction of the disulfide bond responsible for the cytotoxicity of mutant hSOD1.

1.2.4: Quality control mechanisms of misfolded SOD1

As described in the beginning of this chapter, the ubiquitin-proteasome pathway is a component of protein quality control mechanisms within the cell that are implicated in a variety of neurodegenerative diseases, including ALS. In support of the UPP playing a role in ALS pathogenesis, mutant SOD1 is preferentially ubiquitinated and subject to proteasomal degradation at higher levels than the wild-type protein (Stieber et al., 2000; Niwa et al., 2002; Di Noto et al., 2005; Basso et al., 2006). Additionally, dorfin, a RING-finger type E3 ubiquitin ligase, is found in SOD1 inclusion bodies and promotes the degradation of mutant SOD1 *in vitro* (Niwa et al., 2002), and dorfin overexpression improves motor performance and extends the lifespan of hSOD1^{G93A} mice (Sone et al., 2010). Another E3 ubiquitin-ligase of the ubiquitin-proteasome pathway, NEDL1 (NEDD4-like ubiquitin-protein ligase-1), specifically binds to mutant and not wild-type SOD1, and furthermore, is preferentially expressed in the pathologically affected brain and spinal cord (Miyazaki et al., 2004). NEDL1 is also a component of inclusions in lower motor neurons both in fALS patients and mutant SOD1 mice (Miyazaki et al., 2004). Overexpressing human NEDL1 ubiquitously in mouse tissue results in selective motor neuron degeneration, muscular atrophy, and motor deficits (Zhang et al., 2011). Interestingly, the E3 ubiquitin-

ligase CHIP (carboxyl terminus of Hsc70-interacting protein), does not directly ubiquitinate SOD1, but instead promotes ubiquitination of mutant SOD1-interacting Hsp/Hsc70, targeting the entire linked complex for proteasomal degradation (Urushitani et al., 2004). As such, CHIP provides a mechanistic link between chaperone mediated re-folding and proteasomal degradation and could be responsible for mediating protein quality control between these two pathways.

Investigations into proteasome-mediated degradation of mutant SOD1 have yielded conflicting reports. In cell lines that express mutant SOD1, proteasome activity has been variably reported as unchanged, decreased, or increased (Lee et al., 2001; Urushitani et al., 2002; Allen et al., 2003; Aquilano et al., 2003). In tissues from $SOD1^{G93A}$ mice, proteasome activity was reduced specifically in the pathologically affected lumbar spinal cord, but not in the remaining regions of the spinal cord or in unaffected, non-neuronal tissue (Kabashi et al., 2004). In this model, chymotrypsin-like activity was reduced at 45 days of age (presymptomatic) and chymotrypsin-like, trypsin-like, and caspase-like proteasomal activity were all reduced at 75 days of age specifically in the lumbar spinal cord (Kabashi et al., 2004). However, proteasome activity in the entire spinal cord has also been reported to be either increased or unchanged (Cheroni et al., 2005; Puttaparthi and Elliott, 2005). The reasons for these discrepancies are not clear, but these data highlight the importance of assessing specific subtypes of, not just overall, proteolytic activity and of assessing changes between individual regions of the spinal cord.

The distribution of subunits comprising the proteasome varies with the course of disease. As described in the beginning of this chapter, the 20S core is composed of stacked rings of α and β subunits. In conditions of infection and neuroinflammation, the cytokines interferon gamma (INFγ) and tumor necrosis factor alpha (TNFα) stimulate replacement of constitutive proteasome β subunits with inducible subunits (Aki et al., 1994). These inducible subunits, also known as LMP or low molecular weight subunits, are incorporated into the catalytic core to form the immunoproteasome (Bochtler et al., 1999; Groll et al., 1999). Prior to symptom onset, $SOD1^{G93A}$ mice show a decrease in the 20S barrel β5 and β3 subunits specifically in the lumbar spinal cord (Kabashi and Durham, 2006), and this decrease extends to the 20S α subunit and the 19S6b cap subunit in symptomatic animals (Kabashi et al., 2004). Notably, reduction in 20S β5 subunits in pre-symptomatic animals is not accompanied by upregulation of the inducible iβ5 (LMP7) subunit (Kabashi et al., 2008). Late symptomatic animals, however, do demonstrate increased levels of inducible subunits, both iβ5 and 11Sα (the inducible counterpart to the decreased 19S6b subunit) (Kabashi et al., 2004; Cheroni et al., 2005; Puttaparthi and Elliott, 2005). When the spinal cord is examined in its entirety, it is revealed that the inducible 20S subunits LMP2, LMP7, and MECL1 are all upregulated specifically in the CNS of symptomatic $SOD1^{G93A}$ animals, but they are not upregulated in age-matched, wild-type SOD1-overexpressing controls (Puttaparthi and Elliott, 2005). This prompted the hypothesis that the inducible subunits were compensating for the loss of constitutive proteasome subunits. Unexpectedly, crossing LMP2 -/- mice with $SOD1^{G93A}$ animals did not affect either motor function or survival of these animals when compared to standard $SOD1^{G93A}$ animals (Puttaparthi et al., 2007), suggesting that the immunoproteasome does not play an influential role in ALS pathogenesis in this animal model. Alternatively, the presence of the $SOD1^{G93A}$ transgene may be expressed at such

high levels in these animals that subtle alterations in immunoproteasome function are not sufficient to significantly impact motor function or animal survival.

Protein quality control appears to be impaired in ALS, and attempts have been made to upregulate separate components of quality control pathways. Unfortunately, the majority of these experiments have met with limited success, highlighting the complexity of these quality control systems. A clinical trial that may have inadvertently increased proteasome activity in human patients had to be halted early due to loss of lean body mass. In this trial, patients were administered recombinant human ciliary neurotrophic factor (CNTF) with the goal of increasing neuronal trophic support (Miller et al., 1996); it was later demonstrated in rodents that rCNTF increases both 20S proteasome subunit levels as well as proteolytic activity, potentially accounting for the decrease in body mass observed in patients (Jho et al., 2004). Manipulating HSP levels has shown promising preliminary results. Overexpressing Hsp70 in cultured motor neurons expressing mutant SOD1 both increased viability and decreased aggregation of the protein (Bruening et al., 1999). Notably, this effect did not extend to *in vivo* applications; overexpressing Hsp70 in three separate mutant SOD1 mouse models ($SOD1^{G37R}$, $SOD1^{G93A}$, $SOD1^{G85R}$) had no effect on lifespan (Liu et al., 2005). The lack of a protective effect in animals could be due to a need for HSPs to act in concert with each other in more complicated systems. In dorsal root ganglion cultures, HSP70 and HSP27 showed a greater protective effect when overexpressed together than when they were expressed individually (Patel et al., 2005). In rodent models of disease, administration of the drug arimoclomol, a co-inducer of multiple heat shock proteins, significantly extended lifespan, improved limb function, and increased motor neuron survival (Kieran et al., 2004).

Arimoclomol acts by extending activation of heat shock transcription factor 1 (HSF-1), thereby increasing transcription of multiple HSPs (Hargitai et al., 2003). In further support of this model, both pharmacologically activating HSF-1 and expressing a constitutively active form of HSF-1 *in vitro* increased the viability of mutant SOD1-expressing cells in conjunction with increased expression of Hsp70 and Hsp40 (Batulan et al., 2003; Batulan et al., 2006).

The variable expression of heat shock proteins in different cell types may elucidate one of the greater mysteries of ALS, that is, why motor neurons are specifically targeted in this disease. The available evidence suggests that HSPs in motor neurons are less readily upregulated than in other cell types. The small molecular chaperone HSP25 showed increased expression with disease in $SOD1^{G93A}$ mice specifically in astrocytes, but not in motor neurons (Batulan et al., 2003). In a different SOD1 mouse model in which copper cannot be bound by SOD1, HSP25 was upregulated in both neuronal and glial cells in the spinal cord; however, a separate molecular chaperone protein, αB-crystallin, was only upregulated in oligodendroctyes and not motor neurons (Wang et al., 2003). Additional evaluation of both $SOD1^{G93A}$ and $SOD1^{G37R}$ lumbar spinal cords throughout disease demonstrated increased expression of HSP25/27 in neurons and glia in early stages of disease, yet in late disease HSP25/27 was no longer expressed in motor neurons and was instead only found in glial cells (Vleminckx et al., 2002; Maatkamp et al., 2004). Again, up-regulation of αB-crystallin was specific for the relatively protected glia and oligodendrocytes in these mouse models (Vleminckx et al., 2002; Wang et al., 2005). [HSP27 and HSP25 are protein orthologues of the same small molecular chaperone protein, with the mouse form

corresponding to HSP25, and rat and human variants being designated HSP27 (Rogalla et al., 1999)]. It is of note that overexpressing HSP27 in mutant SOD1 mice resulted in delayed disease onset, with no effect on the rate of disease progression (Sharp et al., 2008). The decreased availability of HSPs in motor neurons may reflect a difference in heat shock activation threshold across different cell types. Both exposure to heat shock and excitotoxic levels of glutamate failed to induce HSP70 in motor neurons, although surrounding glia robustly upregulated HSP70 under these conditions (Batulan et al., 2003). Non-steroidal anti-inflammatory drugs (NSAIDs) can enhance the heat shock response by increasing binding of HSF-1 to the heat shock elements in times of stress, thereby amplifying HSP transcription (Jurivich et al., 1992). However, although NSAID exposure lowered the threshold for the heat shock response in glia exposed to heat shock, glutamate, and oxidative stress, the pathologically affected motor neurons were resistant to this effect (Batulan et al., 2005). Therefore, the heat shock response, which is likely protective, is less robust in motor neurons than in other cells of the CNS both in *in vivo* and *in vitro* disease models. It is worth re-emphasizing that heat shock proteins primarily target nascent and soluble, misfolded proteins for re-folding, aggregation or degradation. Therefore, an inability to upregulate heat shock proteins would be detrimental to the quality control of both newly synthesized and misfolded proteins.

1.2.5: Relationship between SOD1 misfolding and cytotoxicity

As previously described, oxidized SOD1 dissociates from a dimeric to an unfolded monomeric state prior to forming aggregates (Rakhit et al., 2004). Therefore, aggregation

necessarily is preceded by protein unfolding and potential misfolding. This has made it difficult to differentiate the effects on cell viability of SOD1 aggregates from soluble, misfolded aggregate precursors, and it is currently unclear if SOD1 aggregation is a toxic or protective mechanism.

Recent studies point to aggregation as being a protective compensatory response to the presence of misfolded, soluble SOD1. In support of this, oligomers and hydrophobic, misfolded SOD1 are present in both $SOD1^{G85R}$ and $SOD1^{G127X}$ mice before disease onset, and are elevated specifically in pathologically affected tissue (Jonsson et al., 2004; Zetterstrom et al., 2007; Wang et al., 2009a). There are conflicting reports detailing when aggregation is initiated in rodent ALS models; however, aggregation is commonly considered to increase most notably at late stages of disease (Watanabe et al., 2001; Wang et al., 2002a; Cheroni et al., 2005; Wang et al., 2009a). The relative dearth of aggregates pre-symptomatically makes their role in disease initiation unlikely. In further support of aggregates not possessing an inherent toxic property, survival of PC12 cells expressing mutant SOD1 was wholly independent of aggregation levels (Lee et al., 2002). However, in a contrasting study, cells exhibiting mutant SOD1 aggregates demonstrated higher cell death than cells expressing a diffuse form of the mutant protein (Matsumoto et al., 2005). Notably, toxicity was also observed in cells lacking aggregates in this study, suggesting that aggregation was not strictly necessary for cell death.

The presence of SOD1 oligomers and protoaggregates prior to disease onset in mutant SOD1 mice and the presence of SOD1 aggregates in otherwise unaffected cells *in*

vitro has led to a focus on soluble, misfolded aggregate precursors as the toxic species in ALS. Mutant SOD1-associated cytotoxicty can be modulated through protein turnover, and upregulation of heat shock proteins has been shown to be neuroprotective in multiple models of ALS (Bruening et al., 1999; Batulan et al., 2003; Kieran et al., 2004; Batulan et al., 2006). Additionally, proteasome function is altered with disease in SOD1 rodent models (Bruening et al., 1999; Urushitani et al., 2002; Puttaparthi et al., 2003). These experiments support the theory that toxicity in ALS is associated with a soluble, misfolded factor and that the aggregates associated with disease reflect sequestration of toxic species and are not toxic themselves. As of yet, no clear correlation has been demonstrated between a mutant's aggregation propensity and the mutant's relative toxicity. Additionally, the biochemical identity of aggregates found in patients is contentious - some studies conclude that SOD1 aggregates are fibrillar, amyloid species, while others claim that ALS-associated aggregates are not amyloid in nature, or that amyloid aggregates may be present, but do not contain SOD1 (Wang et al., 2002b; DiDonato et al., 2003; Chattopadhyay et al., 2008; Kerman et al., 2010). A close examination of these studies reveals that SOD1 only forms amyloid structures in harsh, non-physiological conditions (*e.g.* low pH (DiDonato et al., 2003) or sonication (Stathopulos et al., 2003)), and not within ALS patient tissue. Difficulty in identifying the nature of SOD1 aggregates has contributed both to the difficulty and controversy in interpreting the role that aggregation plays in ALS (Bodner et al., 2006; Outeiro et al., 2007).

Interestingly, the solubility of mutant hSOD1 proteins is increased by co-expression of hSOD1^{WT}, and co-expressing wild-type and mutant hSOD1 correlates with enhanced

toxicity of the mutant protein. *In vitro* studies suggest that hSOD1^{WT} may exacerbate the toxicity of the mutant protein by inhibiting its sequestration into inclusions (Witan et al., 2008; Witan et al., 2009). Rodent disease models further support the view that hSOD1^{WT} enhances mutant SOD1 toxicity – simultaneous expression of wild-type and mutant protein hastens disease onset in SOD1^{G93A}, SOD1^{G85R}, and SOD1^{L126Z} animals, and initiates disease in the otherwise unaffected SOD1^{A4V} mouse (Jaarsma et al., 2000; Deng et al., 2006; Wang et al., 2009b). A separate study on SOD1$^{WT/G85R}$ double transgenic mice indicated that wild-type SOD1 had no effect on toxicity; however, this may be a gene dose effect as the mice in this study had a lower copy number of both transgenes than did the affected SOD1$^{WT/G85R}$ mice (Bruijn et al., 1998). In contrast, inducing hSOD1 aggregation via exposure to hydrophobic lipid vesicles reduced cell viability in culture, but it is not clear if this was due to increased presence of oligomeric aggregate precursors or the aggregates themselves (Kim et al., 2005; Munch and Bertolotti, 2010). The ability of wild-type human protein to both increase mutant SOD1 solubility and to hasten disease in mutant SOD1 rodent models supports a role for soluble SOD1 in pathogenesis. It is possible that increasing the solubility of mutant SOD1 not only exacerbates pathology within individual cells, but also provides a mechanism for disease spread throughout the entire organism.

1.2.6: Does soluble, misfolded SOD1 transfer between cells?

The mechanism by which ALS symptoms present and proliferate throughout the body is not known; the site of symptom onset and rate of progression in patients varies considerably among individual cases. One unifying hypothesis that may fit the majority

(although not all) ALS cases, is the spread of symptoms from a site of focal onset to surrounding contiguous areas (Caroscio et al., 1987). The mechanism of this spread has recently been the subject of intense investigation, with researchers increasingly citing a prion-like mode of disease propagation (Chia et al., 2010; Grad et al., 2011; Munch et al., 2011). In the prion-like model of infectivity, misfolded protein species convert natively folded proteins to an aberrant conformation. Importantly, these newly converted protein species are themselves capable of transmitting the misfolded conformation, and so this process is self-transmissible (Figure 1.6).

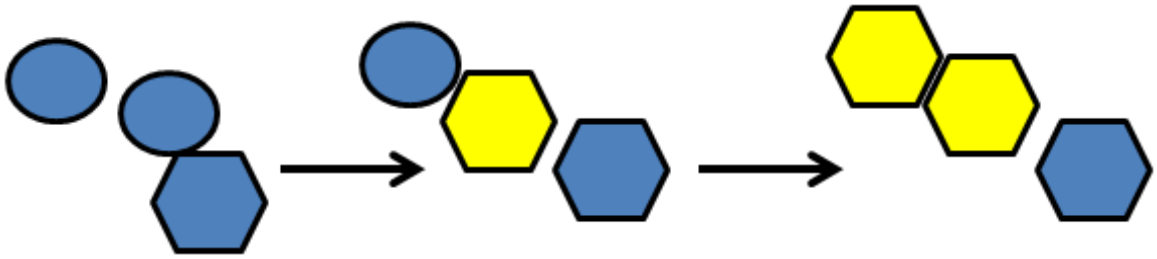

Figure 1.6. Prion-like mechanism of protein misfolding. Natively folded (circle) and misfolded (hexagon) protein molecules interact, resulting in the new conversion of natively folded protein to a misfolded conformer (yellow hexagon). This newly converted (yellow) conformer is then itself capable of inducing the conversion of natively folded protein to the misfolded conformation. In this schematic, newly converted protein molecules, the 'second generation' of misfolded proteins, are represented by the yellow hexagons and the blue hexagons represent the original misfolded protein.

In order for misfolded SOD1 to propagate by cell-to-cell transmission, there must exist an avenue for the protein to exit and enter the cell. Recent studies highlight the secretory ability, and thus potential for cell-to-cell transmission, of this protein. It has long been known that SOD1 can be detected in human CSF (Jacobsson et al., 2001), but its mechanism of cellular export was not clear. Mutant, but not wild-type, SOD1 interactions with chromogranins have been shown to promote the extracellular secretion of SOD1, with the presence of extracellular mutant SOD1 triggering microgliosis and motor neuron death (Urushitani et al., 2006). In contrast, work in the motor neuron cell line, NSC-34, suggests that mutant SOD1 is impaired in its secretion relative to the wild-type protein (Turner et al., 2005). In support of this is the finding that $SOD1^{WT}$ is more abundant in extracellular exosomes than is mutant SOD1 (Gomes et al., 2007). As such, it is not clear if mutant or wild-type SOD1 is more inclined towards secretion, but there is strong evidence that different forms of SOD1 can traverse the cell membrane. In further support of this, exogenous mutant SOD1 aggregates were recently shown to utilize macropinocytosis in entering cells and then to induce, or "seed", the aggregation of mutant, but not wild-type, SOD1 (Munch et al., 2011). Remarkably, these induced, second generation aggregates were able to subsequently seed new aggregates of mutant protein. Therefore, it has been shown that exogenous SOD1 can penetrate cells; however there is still debate regarding the mechanism of this transport, whether mutant or wild-type protein is more inclined towards endocytosis, and the pathogenic effects of exogenous mutant and wild-type SOD1.

In order to evaluate the effects of exogenous SOD1 *in vivo*, the protein has been directly injected into tissue in a few select works. Most notably, both sheep and human ALS

patients (with and without SOD1 mutations) were administered recombinant human wild-type SOD1 intrathecally in order to evaluate the safety of exogenous rhSOD1 (Cudkowicz et al., 1997). Exogenous SOD1 had no deleterious effects in either sheep or humans in this study. There were no observed beneficial effects in the injected human patients, but this study was designed to assess safety, not therapeutic efficacy, and the selected patients had advanced disease at the time of injection. Intraspinal administration of $SOD1^{WT}$ into symptomatic $SOD1^{G93A}$ animals, in contrast, both prolonged survival and decreased cellular vacuolization (Turner et al., 2005); increased SOD1 accumulation was also observed in rodent spinal cord tissue in this study, although the authors did not determine if this was the endogenous $SOD1^{G93A}$ or the exogenous $SOD1^{WT}$ protein. A similar result was observed in a study investigating SOD1 immunotherapy; chronic subcutaneous administration of de-metallated $SOD1^{G93A}$ decreased motor neuron death and delayed disease onset in $SOD1^{G37R}$ mice (Urushitani et al., 2007). Investigations into the acute effects of SOD1 injection on the immune response showed that intracerebral injection of $SOD1^{G93A}$, but not $SOD1^{WT}$, in $SOD1^{G37R}$ mice upregulated pro-inflammatory molecules within 24 hours of injection (Kang and Rivest, 2007) and that intracerebral injection of misfolded mutant, but not wild-type SOD1, caused microglial and astroglial activation within 24 hours in non-transgenic mice (Hernandez et al., 2010). As such, there exists evidence *in vivo* for extracellular SOD1 having functional effects on cell survival, and excitingly, recent *in vitro* work provides a mechanism whereby misfolded SOD1 can spread and propagate protein misfolding in a cell-to-cell transmissible manner.

1.2.7: Can we identify "toxic" misfolded SOD1?

In humans and in animal models, the specific targeting of the motor system by a ubiquitously expressed mutant protein suggests that there may be a difference between mutant SOD1 in motor neurons and SOD1 in non-pathologically affected cells. Specifically, there may be an accumulation of "toxic" SOD1 conformers in pathologically affected tissue. The development of conformationally sensitive antibodies allows researchers to investigate unique, non-native, and potentially toxic, conformers of SOD1. The ability to examine independent conformations of misfolded protein is enormously important because non-native proteins (mutant, oxidized, etc) may assume a myriad of three-dimensional structures, and they may not all be uniformly noxious. Initial attempts to localize misfolded SOD1 relied on the SOD1 Exposed Dimer Interface (SEDI) and the Unfolded SOD1 (USOD) polyclonal antibodies. The SEDI antibody was raised against residues 143-151 of human SOD1 - these residues are located within the dimer interface and are consequently not solvent-accessible in the natively assembled SOD1 homodimer (Rakhit et al., 2007). This antibody labels inclusions within the spinal cord of SOD1 mutant mice as well as SOD1-fALS, but not sALS, patients (Liu et al., 2009). However, it is perhaps a misnomer to state that the SEDI antibody is specific for misfolded SOD1, as its immunoreactive epitope is located at the dimer interface – the most that can be said about this antibody with confidence is that it recognizes monomeric SOD1. The USOD antibody, in contrast, was created against residues 42-48, residues that are normally buried deep within the hydrophobic core of the protein (Kerman et al., 2010). As such, the USOD antibody only recognizes highly unfolded SOD1 protein – this antibody may not detect more subtle

alterations in the three-dimensional state of SOD1. Similar to SEDI, the USOD antibody identified inclusions in SOD1-fALS, but not sALS, spinal cords (Kerman et al., 2010). In attempt to create a more precise system for the detection of misfolded SOD1, the field has increasingly turned to monoclonal antibodies. Two fairly well-characterized monoclonals are the Disease Specific Epitope 1a (DSE1a) and Disease Specific Epitope 2 antibodies (DSE2). The DSE1a antibody is directed against residues 145-151 at the dimer interface, and therefore holds the same limitations as the SEDI antibody in that it cannot truly be said to distinguish misfolded and monomeric SOD1 (Grad et al., 2011). The DSE2 antibody was raised against amino acids 125-142, amino acids that are inaccessible in the natively folded protein. The DSE2 antibody detects inclusions in spinal motor neurons in SOD1 rodent models; to date, no post-mortem human data with this antibody have been reported (Vande Velde et al., 2008). Immunohistochemical studies in post-mortem human tissue utilizing antibodies specific for denatured SOD1 have yielded intriguing results. Inclusions in both sporadic and familial ALS spinal tissue, but not tissue from neurological controls, demonstrate immunoreactivity with these denatured (misfolded?) specific antibodies in immunohistochemical analysis (Forsberg et al., 2010). Interestingly, levels of denatured/misfolded SOD1 in the cerebral spinal fluid did not differ between ALS patients and controls (Zetterstrom et al., 2011). The Julien laboratory has pioneered the study of immunological depletion of misfolded SOD1 using antibodies raised against apo $SOD1^{G93A}$. De-metallated $SOD1^{G93A}$ exists in a disordered conformation, and so antibodies raised against this antigen should recognize misfolded SOD1 (Galaleldeen et al., 2009). Immunization of mutant SOD1 mice with the recombinant $SOD1^{G93A}$ protein or with select "misfolded" specific antibodies extended mouse lifespan while concurrently decreasing misfolded protein load (as detected by the Julien laboratory's in-house antibodies)

(Urushitani et al., 2006; Urushitani et al., 2007; Gros-Louis et al., 2010). We have extensively characterized one of these antibodies, the C4F6 antibody, which appears to be specific not only for misfolded SOD1, but more specifically, for a toxic form of the misfolded protein. As described in chapter 3, the characterization of an antibody specific for toxic protein would facilitate tracking of disease pathology.

1.2.8: Conclusions and hypothesis

Pathological abnormalities in disease-related proteins are a common feature in neurodegenerative diseases, specifically the presence of protein aggregates. Because aggregated intracellular protein inclusions are present in a number of neurodegenerative diseases, there has been a general belief that these aggregates are likely to be toxic, and thus important for disease pathogenesis. However, the presence of aggregated protein species has been shown repeatedly not to temporally correlate with the initiation of toxicity. This prompted the hypothesis that aggregates actually exist as a protective mechanism to sequester away soluble, toxic protein. In this theory, the surface area of toxic protein exposed in the cytoplasm is minimized by isolating misfolded protein into inclusions. This sequestration would reduce aberrant interactions between misfolded, toxic protein, natively folded protein, and cellular organelles.

During my thesis work, I have investigated the relationship between solubility and toxicity of mutant SOD1. As I will discuss more extensively in chapter 5, this topic is too

broad to be satisfactorily resolved by one doctoral dissertation, and so this dissertation focuses primarily on one aspect of this relationship: the correlation between misfolded, soluble protein and cytotoxicity. In chapter 2, I present a novel SOD1 mutation ($SOD1^{C6S}$) in an fALS pedigree, and discuss its relation to solubility. The $SOD1^{C6S}$ mutant is of particular interest because it is located at one of SOD1's four cysteine residues, and aberrant disulfide bonds have been implicated in SOD1 aggregation and ALS pathogenesis. Notably, the $SOD1^{C6S}$ mutant shows incomplete disease penetrance, a long survival time, and diminished aggregation relative to other mutations at this amino acid residue. These features would initially seem to support the hypothesis that aggregated SOD1 is toxic; however it is important to note that the conformation of soluble $SOD1^{C6S}$ is not known, and thus we cannot rule out the possibility that its relatively reduced toxicity is a reflection of a less disordered soluble state. Chapter 3 presents the characterization of a novel antibody, C4F6, that specifically detects toxic, but not benign, misfolded mutant protein. This antibody is specific for pathologically affected tissue in both rodent models of disease as well as in humans presenting with an SOD1 mutation ($SOD1^{A4V}$). Remarkably, this antibody appears to detect easily soluble and not aggregated, insoluble, mutant protein. Finally, in chapter 4, I present data directly investigating the relationship between the relative solubility and toxicity of four different SOD1 mutants in a cell culture model system. I demonstrate that, in the presence of a proteasome inhibitor, the most soluble mutant proteins exhibit the highest degree of toxicity, that this toxicity can be ameliorated through up-regulating the heat shock pathway, and that the heat shock protein rescue effect is independent of conserved aggregates. I further demonstrate that increasing the solubility of mutant SOD1 proteins by co-expressing the wild-type protein proportionately increases each mutant's toxicity. Therefore, an important characteristic that distinguishes toxic from innocuous mutant

SOD1 appears to be solubility. Identifying what renders a mutant protein toxic allows for the development of therapeutics to target that characteristic – this dissertation presents evidence that one of the characteristics of toxic SOD1 that we should be targeting may be its relative solubility.

Chapter 2:

A novel ALS SOD1 C6S mutation with implications for aggregation-related toxicity[1]

[1] This chapter has been re-printed with modification from *Amyotrophic Lateral Sclerosis*, Vol 12, Brotherton et al, A novel SOD1 C6S mutation with implications for aggregation related toxicity and genetic counseling, p. 215-219, © 2011, with permission from Informa Healthcare, License 2932710188699

Abstract

Objective: In this report we describe an ALS family with a novel missense *SOD1* mutation with substitution of serine for cysteine at the sixth amino acid (C6S). This mutation has interesting implications for the role of disulfides in causing disease. **Methods:** After identification of the ALS proband, we examined 17 members of an extended family and performed DNA mutation analysis on 21 family members. The level and activity of SOD1 in C6S carriers and wild-type family members was analyzed in erythrocytes. **Results:** The C6S mutation results in disease with an autosomal dominant mode of inheritance and markedly reduced penetrance. The S6 mutated protein demonstrates high stability relative to the C6-wild-type protein. The specific dismutation activity of S6 SOD1 is normal. **Conclusion:** C6S is a novel fALS associated mutation with reduced disease penetrance, long survival time and a phenotype very different from the other SOD1 mutations reported in codon C6. This mutation may provide insight into the role of SOD1 structural changes in disease.

2.1: Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disease resulting in paralysis and eventual death. The incidence is estimated at 2-3 persons per 100,000 in the Western world. In epidemiological studies, approximately 10% of cases are reported as familial and 90% are sporadic (Siddique et al., 1991). The most common known cause of familial ALS are mutations in the gene *superoxide dismutase1 (SOD1)* (Rosen, 1993). SOD1 is a ubiquitously expressed antioxidant enzyme, composed of two equal 153 amino acid long subunits, each containing a catalytic Cu ion and a stabilizing zinc ion (Tainer et al., 1982). Unusual for a cytosolic protein, each subunit is stabilized by the intrasubunit disulfide bond C57-C146. To date, 155 *SOD1* mutations are associated with disease (Wroe et al., 2008).

Here we provide the first report of a cysteine-to-serine mutation at amino acid 6 (C6S) causing ALS. In addition, this is only the second reported African American family with SOD1 related ALS. This particular mutation is an important addition to the catalog of disease-causing SOD1 mutations since it occurs at one of the 4 cysteine residues that have been intensively studied for their potential role in protein aggregation and SOD1-related toxicity. Even though the C6S mutation has not previously been identified in ALS pedigrees, prior laboratory investigations with this specific mutation have explored its unique biochemical characteristics (Kawamata and Manfredi, 2008).

2.2: Materials and Methods

All protocols and patient/family interactions were approved by the Emory University Institutional review board. All family members interviewed signed informed consent, whether or not they were examined or provided blood samples for genetic testing. All neurological examinations were performed by Dr. Jonathan D. Glass. Analysis of blood samples for mutation analysis and SOD1 activity were performed at Umeå University, Sweden.

SOD1 genotyping

Genomic DNA was extracted from the buffy coat cells with DNA extraction kit NUCLEON BACC2 (GE healthcare) according to the manufacturer's protocol. All five exons and at least 30bp of flanking intronic sequences were amplified with AmpliTaqGold Kit (Applied Biosystems), sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and read in a 3730 DNA Analyzer (Applied Biosystems). The reactions were analysed with SeqScape v 2.5 (Applied Biosystems).

Nomenclature

The novel DNA sequence variant was named c.19 T>A according to the recommendations for nomenclature for the description of sequence variations proposed by human genome variant society, HGVS (www.hgvs.org) (den Dunnen and Antonarakis, 2000). The NCBI

Reference Sequence NM_000454.4 was used as a reference for the coding sequence and the A of the first ATG was used as residue 1. Since the first methionine is removed in the mature SOD1 protein, traditional SOD1 nomenclature ignores this amino acid. Therefore the mutant protein was named C6S using the NCBI Sequence NP_000445.1 as reference and the second encoded amino acid alanine as residue 1 in accordance with traditional SOD1 nomenclature.

Western immunoblots and SOD activity analysis

SOD1 activity and levels were measured in three C6S carriers, and seven wild-type controls. The SOD1 activity in erythrocyte lysates was directly analysed using the KO_2 method (Marklund, 1976). The amount of hemoglobin in the hemolysate was determined with a standard cyanomethemoglobin assay. Activity was measured in triplicate in two samples from each individual. The western immunoblots were performed as described previously (Jonsson et al., 2004). The volumes loaded on the SDS-PAGE gels were adjusted so that the amount of Hb was equal in all samples. The primary antibody (polyclonal rabbit) used was raised against a peptide corresponding to amino acids 57–72 in the human SOD1 sequence. The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and analyzed by Quantity One Software (Bio-Rad). Western immunoblots and quantification were performed twice.

2.3: Results

The proband is a 44 year old African American woman who presented with 1 year of progressive weakness first appearing in the lower extremities. She had brisk tendon reflexes as well as fasciculations and atrophy in the upper and lower extremities. The tongue was normal and she did not have dysarthria. She progressed to being unable to walk, less than antigravity arm weakness and severe respiratory compromise over the next year. She has not developed specific bulbar symptoms or signs.

An extensive family pedigree (figure 2.1) revealed 8 people with ALS, one of whom is currently living (patient 2). This is an African American man who developed weakness of his legs in 1996. The weakness progressed over a decade, and he was confined to a wheelchair in 2007. Examination showed him to have no movement below the waist with weakness of both upper extremities, distal > proximal. The weak muscles were profoundly atrophied, but without fasciculations. Tendon reflexes were reduced and there were no bulbar symptoms or signs. Sensory examination was normal.

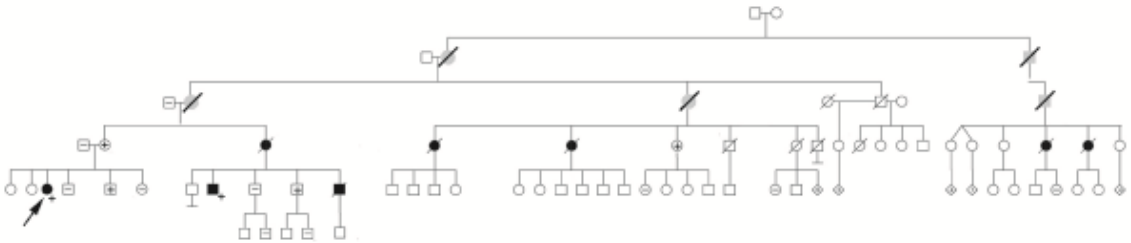

Figure 2.1: Pedigree of ALS history and SOD1 genotypes. Affected individuals are indicated with a black symbol and unaffected with an open symbol. A slashed symbol denotes a deceased individual and the proband is marked with an arrow. '+' = with the C6S mutation, '–' = without the C6S mutation, and gray symbols = obligate carriers.

The other 6 family members with ALS are deceased. One woman was examined by Dr. Jonathan D. Glass about a year after diagnosis, prior to the presentation of the proband. *SOD1* testing was not performed in this case. She was a 67 year old woman with bulbar onset disease associated with severe spasticity, brisk tendon reflexes, and fasciculations. She died 3 years after onset. The other 5 ALS cases were not examined; clinical histories were provided by the family. There were 4 women and 1 man with age of onset between 45 and 66 years. Duration of disease ranged from 3-15 years, with 4 out of 5 showing onset of weakness in the lower extremities. A summary of the clinical characteristics is provided in table 2.1.

Sex	Age at onset	Age at death	Duration (years)	Clinical onset	Other
*F	42		1	Lower extremity	No bulbar
*M	34		14	Lower extremity	No bulbar
*F	66	69	3	Bulbar	Spasticity
M	50	54	4	Lower extremity	Bulbar late
F	66	69	3	Hands/Legs	Bulbar late
F	56	60	4	Lower extremity	No bulbar
F	45	60	15	Lower extemity	No bulbar
F	58	69	11	Lower extremity	Bulbar late

* examined by Jonathan D. Glass

Table 2.1: Clinical characteristics of symptomatic individuals

Mutation analysis in the *SOD1* gene in the proband and patient 2 demonstrated a T>A point mutation at position 19 in the coding sequence resulting in a cysteine to serine substitution at residue 6 (C6S). DNA was not available for the other 6 ALS cases. Nineteen other family members were tested for SOD1 mutations, all of whom were thoroughly examined by Dr. Jonathan D. Glass. Four were found to carry the C6S mutation. All were asymptomatic and had normal neurological examinations. Ages ranged from 47 to 77 years old. Pedigree analysis demonstrated 5 people who were obligate carriers but who died without any symptoms of ALS. The age range for these asymptomatic carriers was 75 to 105 years old. Causes of death were heart disease (2), Alzheimer disease, lung cancer, and "unknown."

Immunoblots of hemolysates from C6S carriers and wild-type family members revealed decreased SOD1 presence in mutant carriers (figure 2.2). Quantification of SOD1 levels and activity are normalized to hemoglobin, as hemoglobin levels per erythrocyte has been shown to exhibit minimal variation between individuals (Andersen et al., 1998). C6S carriers showed 78.3% (± 12.5, SD) total SOD1 present in erythrocytes relative to wild-type controls. If one assumes that 50% of the SOD1 protein present in these samples is wildtype, approximately 28.3% of SOD1 in carrier erythrocytes is C6S. This suggests C6S exhibits high stability. C6S carriers showed 73.5% (±8.2, SD) total SOD1 activity relative to wildtype controls. SOD1 activity did not differ significantly between C6S carriers with and without ALS. Normalized for SOD1 presence, C6S appears to maintain an activity level comparable to wildtype protein (93.9%), as has been previously demonstrated *in vitro* (Kawamata and Manfredi, 2008).

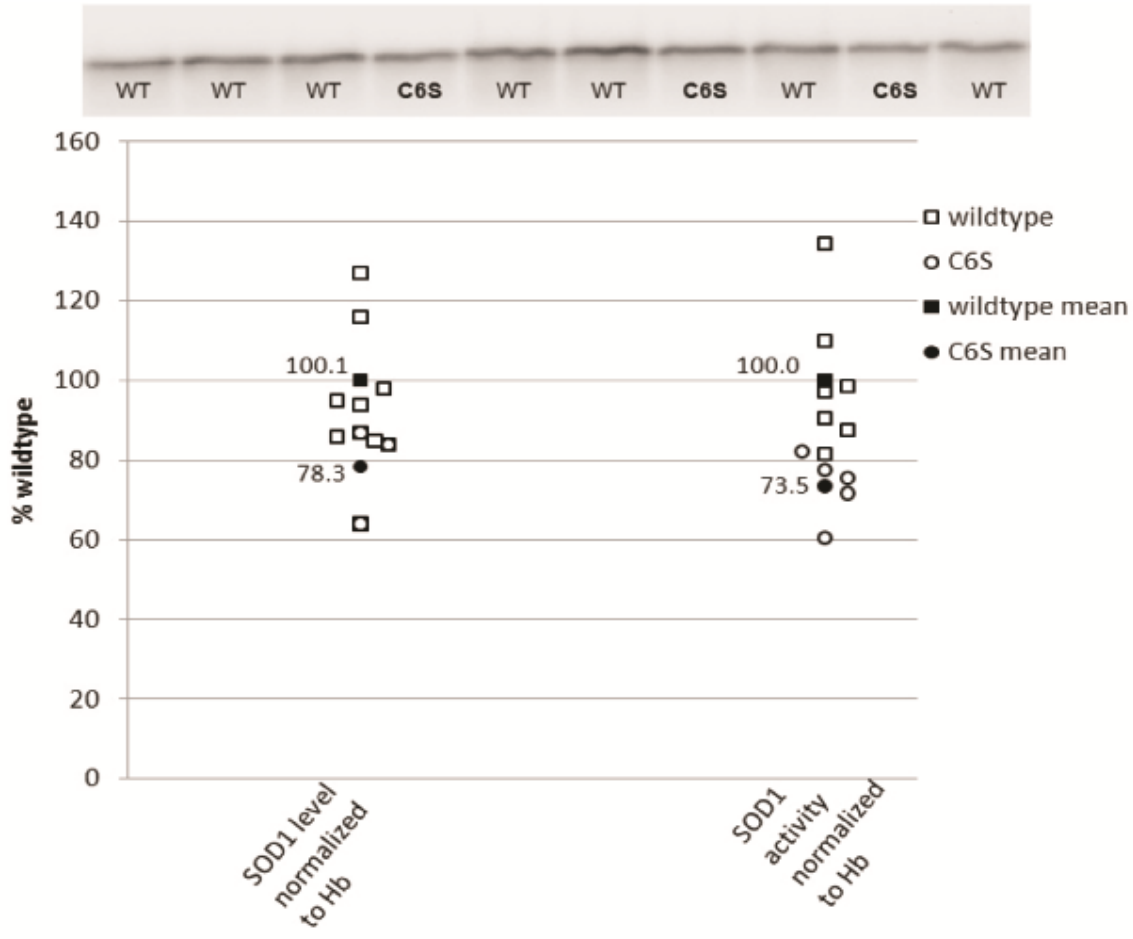

Figure 2.2: Erythrocytes from C6S patients have decreased SOD1 presence.

Hemolysates from C6S carriers and wild-type controls were stained with a polyclonal anti-human SOD1 antibody. SOD1 levels and activity were normalized to hemoglobin and then expressed as percent wild-type. The level of SOD1 in C6S carriers was 78.3% (±12.5, SD) of wild-type controls and the level of SOD1 activity in C6S carriers was 73.5% (±8.2, SD) of wild-type controls. SOD1 activity did not differ between C6S ALS patients and asymptomatic C6S carriers.

2.4: Discussion

This is the first report of the C6S mutation in familial ALS. This family demonstrates an autosomal dominant pattern of inheritance, with striking reduction of disease penetrance. Although 8 family members developed ALS during middle age, there are 5 obligate carriers of the mutation who lived without ALS into their 70's and 80's, including one who died at age 105. Also, there are 4 asymptomatic C6S carriers with normal neurological examinations, 2 of whom are in their 70's. Sequencing of both SOD1 alleles and the lack of a mutation in the father of the proband negate the possibility of recessive inheritance as responsible for incomplete penetrance. Other than reduced penetrance, we can comment that 6 of 8 affected family members had lower extremity onset, and bulbar disease is not prominent in this family (although 1 of 8 presented with bulbar dysfunction). Four affected family members died within 4 years of onset, though 3 lived for > 10 years (including one who is still living). Additionally, this novel SOD1 mutation is of interest because of its manifestation in an African American family, as the majority of fALS documented mutations have been in Caucasian and Asian populations (Wroe et al., 2008).

One proposed mechanism of SOD1 toxicity is aberrant protein aggregation; SOD1 aggregates have been identified in multiple disease models as well as human ALS tissue (Shibata et al., 1994; Shibata et al., 1996a; Shibata et al., 1996b; Johnston et al., 2000; Watanabe et al., 2001). There has been particular interest in the role of cysteines in SOD1 toxicity since aggregation could be partially mediated through aberrant disulfide bonding. Human SOD1 monomers contain four cysteines located at residues 6, 57, 111, and 146. In

the protein's native form, Cys57 and Cys146 form a stabilizing intrasubunit disulfide bond, Cys111 is exposed on the dimer interface, and Cys6 is tightly packed in the β-barrel interior. However, familial ALS SOD1 mutations have previously been reported at cysteines 6 and 146 (www.alsod.org) and cysteine 57 (Andersen et al., unpublished observation) (Morita et al., 1996a; Siddique and Deng, 1996; Kohno et al., 1999). Additionally, murine SOD1 only has 3 cysteines (C111 is replaced with a serine residue), yet mice expressing the G86R mutation in murine SOD1 still develop a highly noxious phenotype (Ripps et al., 1995). This implies no individual cysteine is necessary for SOD1 linked toxicity and weakens support for the idea of aberrant disulfide bonding as causative in disease.

Because of the potential role of cysteines and disulfide bonding in protein aggregation and toxicity of mutant SOD1 proteins, several laboratories have generated cysteine mutants, including the C6S, for *in vitro* studies. These experiments demonstrate that while the highly toxic fALS mutants C6F and C6G are both markedly insoluble, C6S is not prone to aggregation (Cozzolino et al., 2008; Karch and Borchelt, 2008; Kawamata and Manfredi, 2008). If aggregation is a measure of SOD1 toxicity, these data would argue that C6S might be relatively "non-toxic." Our present clinical findings of reduced disease penetrance and long survival time support the notion that the C6S mutant protein may be less toxic than most other SOD1 fALS mutations, including C6G and C6F. The C6S mutant protein was also shown to attenuate the intermolecular disulfide bonding associated with the fALS mutants G85R and G93A (Niwa et al., 2007). Therefore, not only is C6S soluble, it may also increase the solubility of other fALS mutants. This conclusion is tempered by a separate study showing that combining C6S with G93A did not alleviate

insolubility, intermolecular linkages, or toxicity associated with the G93A mutation (Cozzolino et al., 2008). It has recently been questioned whether or not all reported mutations in the SOD1 gene are pathogenic (Felbecker et al., 2010). However, we propose that the C6S mutation is the cause of ALS in this pedigree: the C6S mutation co-segregates with disease and is found in two affected cousins (being cousins, the likelihood that they would share an allele is 12.5%). Further indirect support for a causative role of C6S in ALS is that other mutations have been found in C6; the aforementioned C6G and C6F are both notable for their manifestation as highly penetrant and with rapidly progressive disease course although the reported pedigrees were small (Morita et al., 1996b; Kohno et al., 1999). The C6S mutation, in contrast, results in a phenotype of variable progression and incomplete penetrance. It is of note that serine shares high structural similarity with cysteine, and is actually its closest homolog among the amino acids. The wildtype-like dismutase activity and relative stability of the 6S mutant may well be a reflection of this structural similarity.

In conclusion, this novel fALS mutation provides the unique opportunity to investigate the clinical questions of penetrance and phenotypic variability as well as the biochemical issues of solubility and structural stability.

Chapter 3

Tracking SOD1-associated toxicity: localization of a subset of fALS SOD1 protein to pathologically affected tissues[2]

[2] This chapter has been re-printed with modification from *PNAS*, Vol 109, Brotherton et al, Localization of a toxic form of superoxide dismutase 1 protein to pathologically affected tissues in familial ALS, ©2012 National Academy of Sciences, USA.

Abstract

Mutations in the gene encoding superoxide dismutase 1 (SOD1) account for about 20% of cases of familial amyotrophic lateral sclerosis. It is not known how the mutant protein causes disease, or why only a subset of cell types (motor neurons) are targeted. The aggregation and misfolding of mutant SOD1 are implicated in disease pathogenesis in both animal models and humans. We used a monoclonal antibody, C4F6, that specifically reacts with mutant and/or 'misfolded' SOD1, to investigate the regional distribution of mutant SOD1 protein in rodent and human tissues. C4F6 reacted only with mutant SOD1 and showed remarkable selectivity for disease-affected tissues and cells. Tissue not affected by disease but containing high levels of mutant protein (sensory neurons), did not stain with C4F6. Additionally, C4F6 intensely stained some motor neurons while leaving adjacent motor neurons unstained. Although C4F6 was generated against the G93A SOD1 mutant, it also recognized other SOD1 mutants. In human autopsy tissues from patients carrying SOD1 mutations, C4F6 identified skein-like intracellular inclusions in motor neurons, similar to those seen in rodents, and again stained only a subset of motor neurons. In spinal cords from patients with sporadic ALS, other neurodegenerative diseases, and normal controls, C4F6-immunoreactive inclusions were not detected, but the antibody did reveal diffuse immunostaining of some spinal motor neurons. The ability of C4F6 to differentiate pathologically affected tissue in mutant SOD1 ALS rodent models and humans, specifically motor neuron populations, suggests this antibody may recognize a 'toxic' form of the mutant SOD1 protein.

3.1: Introduction

Amyotrophic lateral sclerosis is a progressive, adult-onset motor neuron disease with an incidence of 1-2 persons per 100,000 (Worms, 2001; Logroscino et al., 2010). ALS typically causes death within 3-5 years of diagnosis; there is no cure and treatment options are very limited (Millul et al., 2005b; Beghi et al., 2008; Logroscino et al., 2008b). Ten percent of ALS cases are familial (fALS), with 20% of fALS being linked to mutations in the homodimeric protein, Cu/Zn superoxide dismutase 1 (SOD1) (Siddique et al., 1991; Rosen et al., 1993; Chio et al., 2008). In animal models and in humans carrying SOD1 mutations, mutant SOD1 is 'toxic' only to motor neurons, and only after it has been present for an extended period of time (aging), leading to the important questions of how mutant SOD1 exerts its toxicity, and why motor neurons are particularly sensitive to the pathogenic process.

Mutant SOD1 has a high propensity to undergo conformational changes and aggregation when stressed *in vitro*, and SOD1 aggregates and misfolded aggregate precursors have been localized specifically to pathologically affected tissues in animal models of disease, suggesting that SOD1 conformational changes may contribute to the toxicity of the mutant protein (Gaudette et al., 2000; Johnston et al., 2000; Puttaparthi et al., 2003; Rakhit et al., 2004; Puttaparthi et al., 2007; Zetterstrom et al., 2007). It has been further demonstrated that conformational changes in wild-type SOD1 can be induced through oxidation and/or

metal depletion, and that these conformers of the wild-type protein are also toxic in cellular models (Beckman et al., 1999; Rakhit et al., 2002; Abou Ezzi et al., 2007; Banci et al., 2007). Additionally, misfolded wild-type SOD1 has been identified both in neurons and glia of sporadic ALS (sALS) patients (Bosco et al., 2010; Forsberg et al., 2011) and misfolded wild-type SOD1 derived from sALS tissue inhibits fast axonal transport to a degree reminiscent to that of mutant SOD1 (Bosco et al., 2010). Therefore, misfolded SOD1 may provide a mechanistic link between sporadic and familial forms of ALS.

Several immunological tools have been developed in an attempt to isolate the "toxic" SOD1 species. Antibodies have been created against the SOD1 dimer interface, against residues in the hydrophobic core, and against specific SOD1 mutant proteins (Chakrabartty et al., 2007; Urushitani et al., 2007; Robertson et al., 2009; Gros-Louis et al., 2010). We present here further characterization of the C4F6 antibody, which is known to react specifically with mutant human SOD1 (hSOD1) proteins (Urushitani et al., 2007; Bosco et al., 2010). The antibody was generated against the apo (metal-free) form of $hSOD1^{G93A}$, which exhibits a disordered conformation (Galaleldeen et al., 2009). Immunization with recombinant $hSOD1^{G93A}$ protein increases the lifespan of $hSOD1^{G37R}$ mice, while simultaneously decreasing C4F6 detection of mutant hSOD1 protein, suggesting that C4F6 recognizes 'toxic' mutant SOD1 (Urushitani et al., 2007). It has been difficult to identify the precise C4F6 immunoreactive epitope, however exon 4 of the SOD1 protein is known to be crucial for C4F6 recognition (Bosco et al., 2010). We provide a detailed characterization of this antibody's immunoreactivity both *in vitro* and in mouse, rat and human tissues. We demonstrate a unique pattern of C4F6 immunoreactivity that is selective for clinically and

pathologically affected tissues in transgenic ALS rodent models and in humans with SOD1 mutations, and characterize the biochemical nature of SOD1 protein that is immunoreactive with C4F6. These new data indicate that C4F6 recognizes a subset of mutant hSOD1 that is instrumental in the clinical and pathological pattern of motor neuron disease.

3.2: Materials and Methods

Animals

All animal studies were approved by the Emory University Animal Care and Use Committee. Transgenic mice overexpressing $hSOD1^{G93A}$ (B6SJL-Tg(SOD1-G93A)1Gur/J) and $hSOD1^{WT}$ (B6SJL-Tg(SOD1)2Gur/J) were initially purchased from the Jackson Laboratory, and were maintained on the C57/BL6 background. In our colony, symptom onset typically occurs at about 90 days of age, and end-stage (defined as when the animal can no longer right itself within 30 seconds when placed on its back) occurs at about 135 days. $hSOD1^{G93A}$ rats were initially obtained from Charles River Laboratory, and the colony was maintained by crossing with Sprague-Dawley rats. All animals were housed in microisolator cages with *ad libitum* access to food and water.

Cell culture and transfections

Chinese Hamster Ovary (CHO) cells were maintained in Gibco F-12 Hams media (11765) supplemented with L-glutamine, penicillin/streptomycin, and fetal bovine serum. Cells were

transiently transfected at 95% confluency with Lipofectamine 2000 according to manufacturer protocols.

Immunohistochemistry

Antibodies used were a pan-SOD1 sheep polyclonal antibody (1:800, Calbiochem), and C4F6 (JPJulien, Laval University, 1:100 on rodent tissue and 1:50 on human tissue). Paraffin embedded tissue blocks were cut at 8-10 μm. Slides were re-hydrated through a gradient of 2 x 5 minutes in xylene, 3 x 3 minutes in 100% EtOH, 3 x 3 minutes in 95% EtOH, 3 x 3 minutes in 70% EtOH, and then rinsed in ddH_2O, followed by Tris Buffer Saline (TBS). For human tissue, antigen retrieval was performed at this stage; no antigen retrieval was necessary with rodent tissue. For antigen retrieval, tissue was microwaved 9 x 2 minutes in sodium citrate buffer (pH 6) with the sample being closely monitored to ensure that the tissue never dried out. The sample was then left at room temperature still in buffer for 1 hour to allow antigenic sites to reform, and then gently rinsed in ddH_2O before being rinsed 3 x 3 minutes in TBS. From this point on, the rodent and human protocols are the same. The samples were incubated for 10 minutes in 3% H_2O_2, then rinsed 5 x 5 minutes in TBS. Samples were incubated in an avidin pre-block solution in TBS composed of 10 μg/μl avidin, 4% normal goat serum, and 0.1% Triton-X. Following 3 x 5 minute TBS washes, samples were incubated at 4°C overnight in primary antibody; primary antibody buffer was made in TBS and contains 50 μg/μl biotin and 2% normal goat serum. Following overnight incubation in primary antibody, tissue was rinsed 4 x 5 minutes in TBS and incubated with biotinylated secondary antibody in 2% normal goat serum for 1 hour at room temperature. Tissue was rinsed 4 x 5 minutes and incubated for 1 hour in Vector Elite ABC kit, following

manufacturer instructions. Slides were then rinsed 5 x 5 minutes and developed in diaminobenzidine (Sigma D4293) following manufacturer instructions, rinsed extensively, dehydrated and cover slipped.

Immunocytochemistry

Cells were fixed for 20 minutes in 4% paraformaldehyde, and rinsed extensively in TBS. Non-specific binding was then blocked for 2 hours at 4°C in 10% normal goat serum and 0.4% Triton-X in TBS. Cells were incubated overnight in primary antibody in block buffer at 4°C; antibody concentration for C4F6 was 1:200 and for the human SOD1 specific antibody (mouse monoclonal, Sigma) was 1:500. The next day, cells were rinsed 3 x 10 minutes in TBS and incubated with rhodamine goat anti-mouse secondary antibody for 1 hour at room temperature. Cells were rinsed extensively and nuclei stained with DAPI prior to coverslipping in Vectashield (Vector Labs) to preserve fluorescence.

Differential Extraction of SOD1 protein from tissue

Spinal cord tissues from hSOD1^{G93A} mice were homogenized in progressively harsh conditions to determine the relative solubility of the mutant SOD1 protein that reacted with the C4F6 antibody. First the tissue was homogenized in detergent-free buffer composed of 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 2 mM Na_3VO_4, 6 mM NaF, and a Roche Mini Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) using a glass on glass homogenizer apparatus. Samples were then centrifuged at 16,000 x *g* for 10 minutes at 4°C, and the supernatant (detergent–free, soluble protein)

removed. The remaining pellet was rinsed 3 times in the homogenization buffer, and then re-suspended in homogenization buffer containing 1.5% NP-40 before being spun at 16,000 x *g* for 10 minutes to isolate the NP-40 soluble protein. The pellet was washed 3 times, and re-suspended in buffer containing 1.5% NP-40 and 2% SDS, spun at 16,000 x *g* and the SDS soluble protein was removed. The pellet was again washed 3 times and re-suspended in buffer containing 1.5% NP-40, 2% SDS, and 6 M urea. Following being spun at 16,000 x *g* for 10 min at 4°C, the supernatant (urea soluble protein) was removed, and the pellet was rinsed 3 times before being re-suspended in the same buffer and sonicated to solubilize any remaining protein. Quantification was performed using ImageJ densitometry software, and the percent C4F6 protein in each solubility condition was calculated as the combined dorsal and ventral C4F6 optical density (OD) value for that solubility condition over total C4F6 OD.

Immunoblotting

Protein samples in 2x Laemmli sample buffer (Bio-Rad Catalog # 161-0737 + 5% βME) were heated at 85°C for 5 minutes, separated by SDS-PAGE in a 4-20% gradient gel, and transferred to PVDF membrane prior to blocking 1 hour in 5% milk at room temperature. Membranes were incubated overnight at 4°C with primary antibody (Calbiochem pan-SOD1 sheep polyclonal, 1:1,000 or C4F6 mouse monoclonal 1:500). The following day, membranes were rinsed 3 x 5 minutes in PBS/Tween, and then incubated for 1 hour with near-infrared (IR) Dye conjugated secondary antibody. The membrane was washed extensively and visualized with an Odyssey scanner (LI-COR Biosciences). Protein was normalized by equivalent volume in the HIC and differential extraction techniques and by

equivalent total protein concentration in all other immunoblots. Samples for native immunoblot analysis were prepared as for denaturing immunoblot, except there were no detergents or reducing agents present in either the homogenization buffer (PBS) or Laemmli sample buffer and the samples were not heated prior to separation on a non-denaturing, 12% Tris/Glycine gel. Human SOD1 purified from erythrocytes was purchased from Sigma (Catalog # S9636).

Hydrophobic Interaction Chromatography (HIC)

HIC protocol was adapted from (Zetterstrom et al., 2007). Spinal cords from hSOD1^{G93A} mice were weighed and ultra sonicated in ice-cold PBS (25 ml/gram tissue) containing 20 mM iodoacetamide and Roche Mini Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Homogenates were spun at 16,000 x *g* for 25 minutes at 4°C, and the supernatants removed for HIC analysis. HIC columns were packed with 1 mL Octyl-Sepharose CL-4B (GE Health Care, Uppsala, Sweden) and were allowed to empty spontaneously with gravity. 250 μL of sample was loaded onto the column, followed by 500 μL PBS. After 15 minutes binding time columns were eluted two times with 2.5 mL PBS, and then twice more with 2.5 mL PBS diluted 10 times. The columns were then eluted with 2.5 mL elution buffer, composed of 4% SDS in 50 mM Tris-HCl, pH 6.8 followed by 2 mL PBS. Eluted fractions were separated on an electrophoretic gel and probed for C4F6 and pan-SOD1 immunoreactivity as outlined above.

Classifying C4F6 immunoreactivity

Human spinal cords were examined and rated for C4F6 immunoreactivity by 3 independent examiners blinded to the clinical diagnoses of the patients. Scoring was as follows: no staining, diffuse staining in a minority of neurons, diffuse staining in the majority of neurons, or skein-like intracellular inclusions. Discrepancies in scores between reviewers were resolved by consensus, with all examiners viewing the tissues together. Autopsy tissue examined included 25 sporadic ALS cases, 2 fALS without SOD1 mutations, 3 $SOD1^{A4V}$ mutants, and 23 non-ALS controls. Controls were 18 cases with other neurological diseases (OND) and 5 cases without neurological disease. Primary diagnoses in OND cases were 10 Alzheimer Disease, 2 dementia with Lewy bodies, 1 Parkinson's Disease, 1 tauopathy (with possible AD), 1 progressive supranuclear palsy, 1 neuronal intermediate filament inclusion disease, and 2 diagnoses of frontotemporal lobar degeneration without motor neuron disease.

3.3: Results

The C4F6 antibody selectively stained pathologically affected cell populations (i.e. motor neurons) in both mouse and rat ALS disease models. C4F6 was immunoreactive only with motor neuron populations in these models; no immunoreactivity was observed in secondary sensory neurons in the dorsal horn or in primary sensory neurons in the dorsal root ganglia (figure 3.1A, I). Immunoreactivity was not uniformly observed across the ventral horn; some motor neurons were intensely stained by C4F6, while neighboring

neurons with similar morphologies remained unstained (figure 3.1E, F). Ventral roots exiting the spinal cord were C4F6-immunoreactive, whereas the corresponding dorsal roots did not stain with C4F6 (figure 3.1G, H). C4F6 staining of hSOD1^{G93A} spinal cord was observed throughout the disease course (pre-symptomatic through end-stage); the distribution of staining expanded with aging, but remained relatively specific for pathologically affected cell populations (figure 3.S1). This selectivity for disease-targeted tissues was not due to varying amounts of hSOD1 in these tissues, as staining with a pan-SOD1 antibody demonstrated high levels of SOD1 in clinically unaffected, non-C4F6-immunoreactive tissue (figure 3.1B, J). Furthermore, C4F6 did not stain in transgenic animals overexpressing wild-type hSOD1, even though hSOD1 was present at high levels in these animals (figure 3.1C, D).

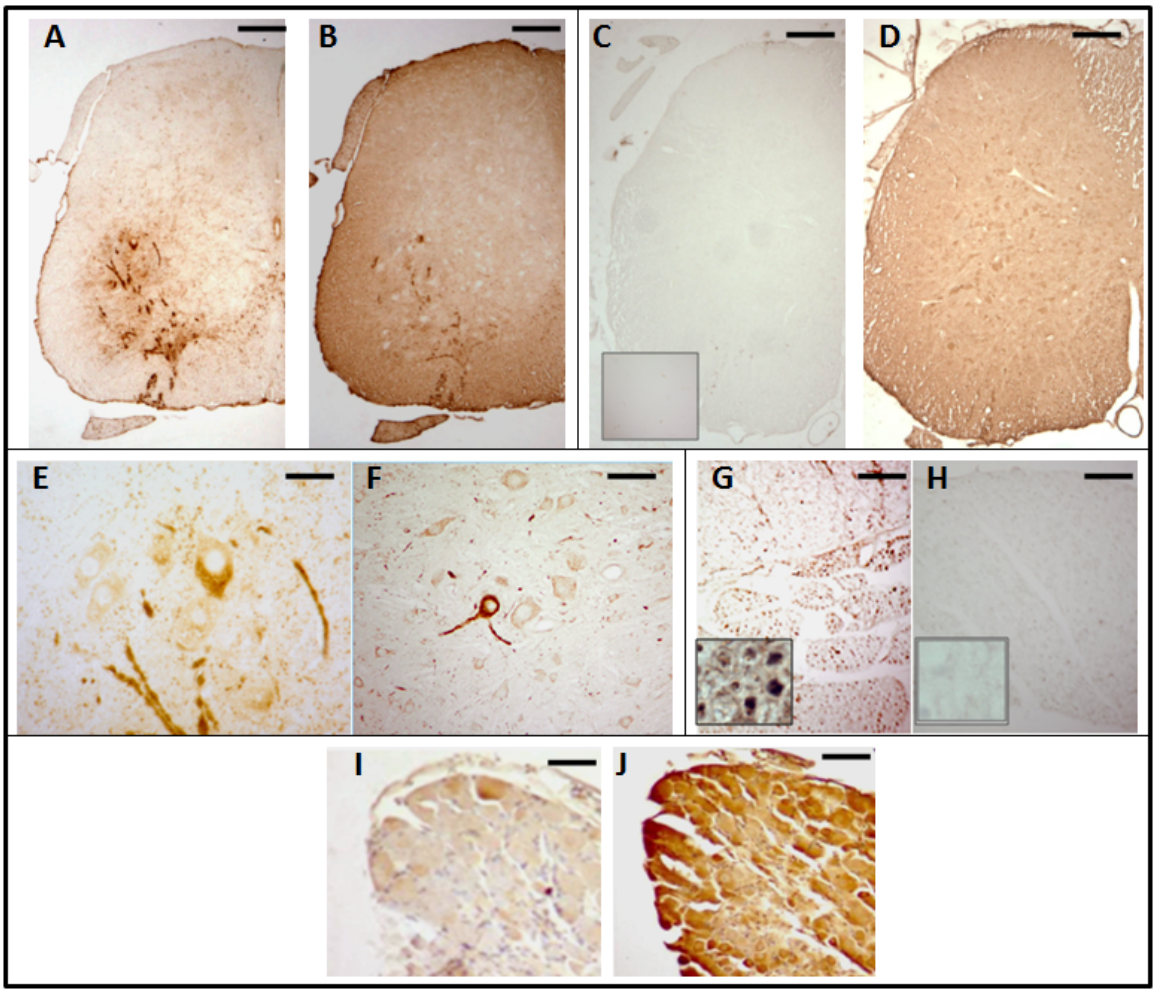
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Figure 3.1: C4F6 is selective for pathologically affected tissue types in ALS rodents. Spinal cord sections from 47 day hSOD1^{G93A} (A, B) and 215 day hSOD1^{WT} (C, D) mice. A and C are stained with the C4F6 antibody and B and D are stained with the pan-SOD1 antibody. (A) Note C4F6 staining of anterior horn cells and ventral root in the ventral spinal cord and the relative lack of staining in the dorsal spinal cord and dorsal root in hSOD1^{G93A} tissue. C4F6 did not stain the spinal cord from hSOD1^{WT} (C). The inset in panel C further demonstrates this lack of immunoreactivity with a high power image of the hSOD1^{WT} ventral horn stained with C4F6. Staining with the pan-SOD1 antibody demonstrates the diffuse presence of human mutant (B) and wild-type (D) hSOD1 protein. Spinal cord from SOD1 ^{G93A} mouse (E) and rat (F) demonstrates C4F6 stains only a subset of motor neurons within pathologically affected tissue. Furthermore, C4F6 stains rat hSOD1^{G93A} ventral root (G) but not dorsal root (H). The insert in panel G shows a high power image of ventral root axons showing staining of some, but not all axons; the insert in panel H demonstrates minimal staining of dorsal root axons. I-J demonstrates C4F6 does not recognize mutant SOD1 protein in non-affected SOD1^{G93A} dorsal root ganglia (I), despite the presence of human mutant SOD1 identified with the pan-SOD1 antibody (J). Scale bars: E = 50 μm, F-J = 100 μm, all other scale bars = 200 μm. Independent experiments were performed a minimum of three times.

Interestingly the selectivity of C4F6 for clinically and pathologically affected tissues by immunohistochemistry did not extend to denaturing immunoblot. When the SOD1 protein was denatured through SDS-PAGE, C4F6 recognized mutant hSOD1 protein in dorsal (sensory) spinal cord (figure 3.2A) as well as in ventral spinal cord. C4F6 remained specific for mutant protein in immunoblots and did not stain wild-type hSOD1 (figure 3.2B). However, when SOD1 protein was separated on a native, non-denaturing gel, C4F6 again only detected mutant protein in the ventral horn (figure 3.S2).

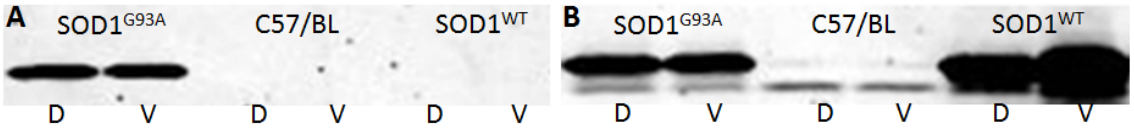

Figure 3.2: Both disease affected and unaffected hSOD1^{G93A} tissue is C4F6-immunoreactive when denatured through SDS-PAGE. C4F6 (A) is immunoreactive with both denatured dorsal (D) and ventral (V) hSOD1^{G93A} spinal cord. C4F6 does not react with dorsal or ventral cord from either C57/BL6 or hSOD1^{WT} mice. Probing with a pan-SOD1 antibody (B) demonstrates hSOD1 presence; mouse SOD1 (lower band) runs slightly faster than human SOD1 on SDS-PAGE. Independent experiments were performed a minimum of three times.

Because insoluble aggregates of mutant SOD1 proteins are hypothesized to be toxic (Matsumoto et al., 2005; Stojanovic et al., 2005), and because we suspect that C4F6 reacts with a toxic form of mutant SOD1 protein, we addressed the relative solubility of C4F6-immunoreactive protein using differential solubility extraction. Spinal cords from late-stage hSOD1^{G93A} animals were homogenized in increasingly harsh solvents, and progressive fractions were probed for total SOD1 protein and for C4F6 immunoreactivity. The majority of C4F6-immunoreactive protein was soluble in buffer lacking detergent, with very little requiring harsh detergents for solubility (figure 3.3, top panel). Quantification of the C4F6 immunoreactivity in each solubility condition reveals that approximately 59% of total C4F6 immunoreactive protein was soluble in the complete absence of detergent, and approximately 27% of total C4F6 immunoreactive protein was soluble with the addition of the relatively mild detergent, NP-40. These results suggest that the protein species preferentially recognized by C4F6 is not insoluble, aggregated protein. Indeed, C4F6 did not recognize the most insoluble hSOD1 species that was detected by the pan-SOD1 antibody (figure 3.3, bottom panel).

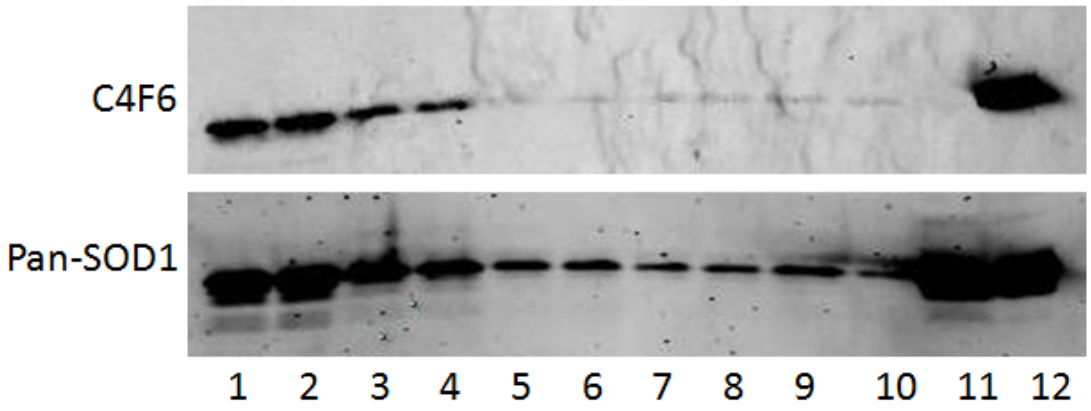

Figure 3.3. C4F6-immunoreactive protein is easily soluble. Dorsal (odd numbered lanes) and ventral (even numbered lanes) spinal cord from an end-stage hSOD1^{G93A} mouse extracted in buffer without detergent (lanes 1,2), with 1.5% NP-40 (3,4), with 1.5% NP-40 and 2% SDS (5,6), with 1.5% NP-40, 2% SDS, and 6 M urea (7,8), and the remaining pellet (9,10). Lane 11 is 215 day hSOD1^{WT}, lane 12 is 70 day hSOD1^{G93A}. Protein was separated through denaturing SDS-PAGE, and transferred to PVDF membrane for immunoblot. Top panel probed with C4F6 and bottom panel probed with a pan-SOD1 antibody. Note the relatively equal amounts of mutant hSOD1 protein in dorsal vs. ventral spinal cord, in contradistinction to the C4F6 immunoreactivity seen in tissue sections. Independent experiments performed in duplicate.

Because mutant SOD1 proteins have a tendency to misfold (Shaw et al., 2006; Durazo et al., 2009; Hayward et al., 2009), we investigated whether the C4F6-immunoreactive hSOD1 showed evidence of being misfolded using hydrophobic interaction chromatography (HIC). HIC operates on the principle that a natively folded protein will shield its hydrophobic core from the hydrophilic environment, whereas misfolded protein will have a greater propensity to expose hydrophobic residues. HIC provides a technical tool to isolate misfolded proteins, and has been used to demonstrate that soluble, misfolded forms of hSOD1 are present in fALS mouse spinal cords prior to the onset of symptoms (Zetterstrom et al., 2007). We hypothesized that if C4F6 recognizes a misfolded form of SOD1, this form would be selectively enriched in the hydrophobic (misfolded) fraction. However, HIC and subsequent immunoblot of $hSOD1^{G93A}$ spinal cords demonstrated that the majority of C4F6 immunoreactive protein was present in the non-hydrophobic fractions, although some C4F6 immunoreactive protein was identified as hydrophobic (figure 3.4).

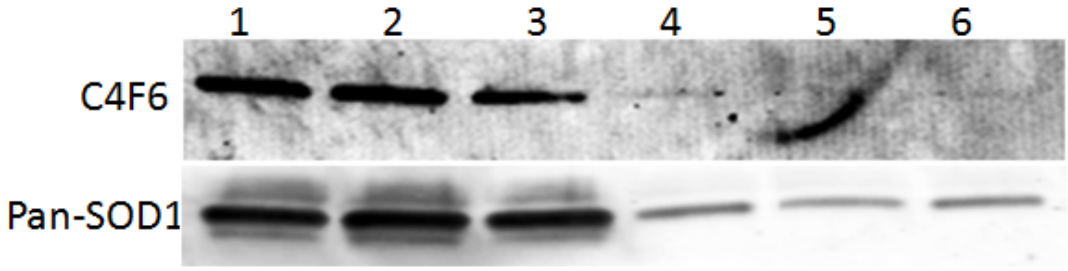

Figure 3.4. C4F6 does not robustly detect hydrophobic SOD1. Spinal cord from 135 day hSOD1^{G93A} mouse separated by hydrophobic interaction chromatography and visualized through immunoblot following denaturing SDS-PAGE. C4F6 (top panel) detects the input (lane 1) and flow-through not caught on the column (2, 3) but only weakly detects hydrophobic protein eluted off the column (4, 5, 6). A pan-SOD1 antibody (bottom panel) demonstrates hydrophobic SOD1 is present in the eluate. Blot representative of duplicate experiments.

Even though the C4F6 antibody was raised against hSOD1^{G93A} mutant protein, it also recognizes linearized hSOD1^{G37R} mutant protein (Urushitani et al., 2007), suggesting that the reactive epitope(s) encompass more than the G93A point mutation. To further investigate the reactivity of C4F6 for other SOD1 mutants, CHO cells were transiently transfected with plasmids for A4V, G37R, and G93C human SOD1. C4F6 stained cells expressing hSOD1^{A4V} and hSOD1^{G37R}, but did not stain cells expressing hSOD1^{G93C} (figure 3.5). As in the mice, C4F6 did not stain CHO cells expressing hSOD1^{WT}, whereas the human-specific antibody identified hSOD1 in all transfection conditions. The ability of C4F6 to detect some non-G93A mutant SOD1 proteins confirms previous data showing that the presence of mutant human SOD1 protein, possibly 'toxic' and/or misfolded SOD1, is sufficient for C4F6 recognition (Urushitani et al., 2007). However, not all mutants (i.e. hSOD1^{G93C}) are recognized by C4F6. A possible explanation for the inability of C4F6 to recognize G93C is that the 93C mutation is able to form a non-native disulfide bond that might block the epitope, though we yet have no evidence to support this hypothesis. As ovary cells are not typically affected in ALS, C4F6 immunoreactivity in transiently transfected CHO cells may at first seem surprising. However, an isolated cell line may not respond to a stressor in the same manner as cells *in vivo,* and the stress of transient transfection may also increase cell vulnerability.

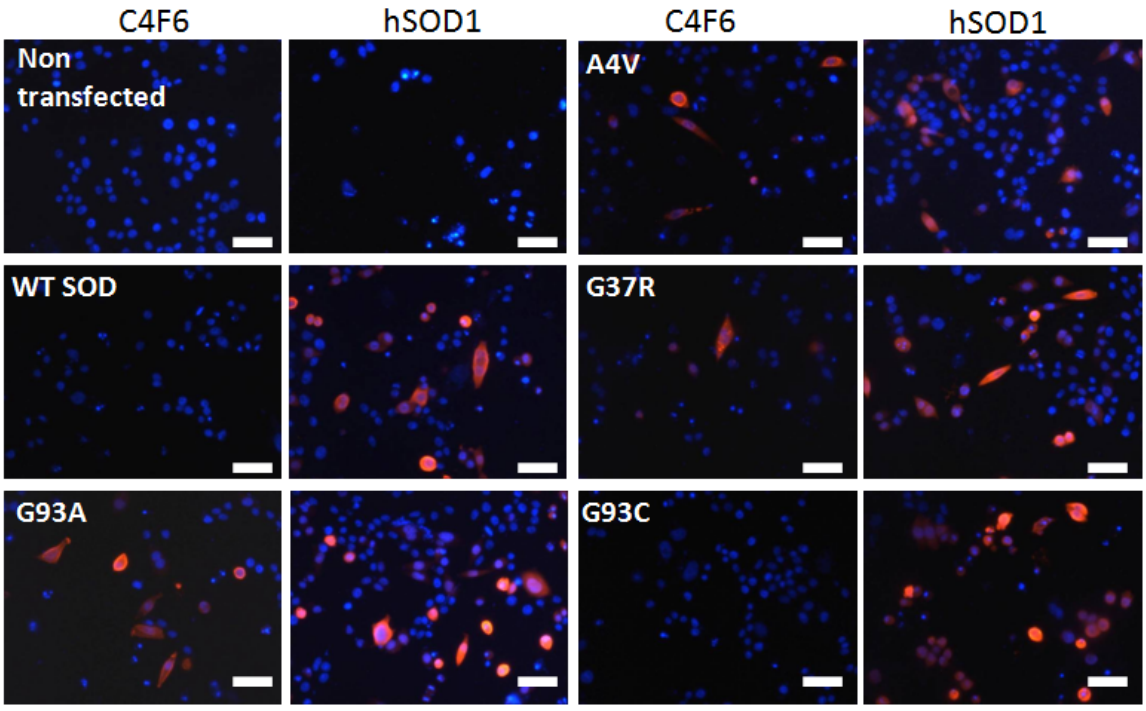

Figure 3.5: C4F6 detects non-G93A hSOD1 mutants. CHO cells transiently expressing hSOD1^{G93A}, hSOD1^{A4V}, hSOD1^{G37R}, hSOD1^{G93C}, and hSOD1^{WT} were stained with C4F6 (red), or a human SOD1 specific antibody (red) in order to demonstrate hSOD1 expression. DAPI denotes nuclei (blue). C4F6 detected hSOD1^{G93A}, hSOD1^{A4V}, and hSOD1^{G37R} but not hSOD1^{G93C} or hSOD1^{WT}. Scale bar = 50 μm. Independent experiments were performed three times.

Finally, we investigated the immunoreactivity of C4F6 in human tissues, asking whether the antibody would show similar specificity for motor neurons as was demonstrated in the rodent tissues. In addition, there was a question of whether C4F6 could detect SOD1 even in cases of sporadic ALS, as was demonstrated previously (Bosco et al., 2010). We examined 30 ALS spinal cords, and 23 spinal cords from others dying with or without other neurological diseases. Of the 30 ALS patients, three harbored an SOD1 mutation (A4V), and two patients had familial ALS without an SOD1 mutation. Staining with the C4F6 antibody showed distinct, skein-like intracellular inclusions in motor neurons only in the A4V ALS spinal cords (figure 3.6, top row). This pattern of staining was not seen in spinal motor neurons from sALS, non-SOD1 fALS, other neurological disease or non-neurological controls (figure 3.6, bottom row). However, we saw diffuse staining of motor neurons in all cases, which was similar in ALS patients and controls (Table 3.1). The similarity of diffuse neuronal staining in non-SOD1 ALS patients, other neurological disease, and non-neurological controls was not changed by varying the staining methodology. Omitting the antigen-retrieval step from the protocol reduced staining in all cases, but did not change the interpretation of the primary data. In addition, replacement of the C4F6 antibody with normal mouse serum resulted in diffuse staining in some motor neurons, similar to that seen with C4F6 in control tissues (figure 3.S3). This diffuse staining therefore may be non-specific background or reaction with lipofuscin. As is typical in autopsy specimens from adults, many motor neurons showed the granular perikayal staining that is typical of lipofuscin pigment (figure 3.S3).

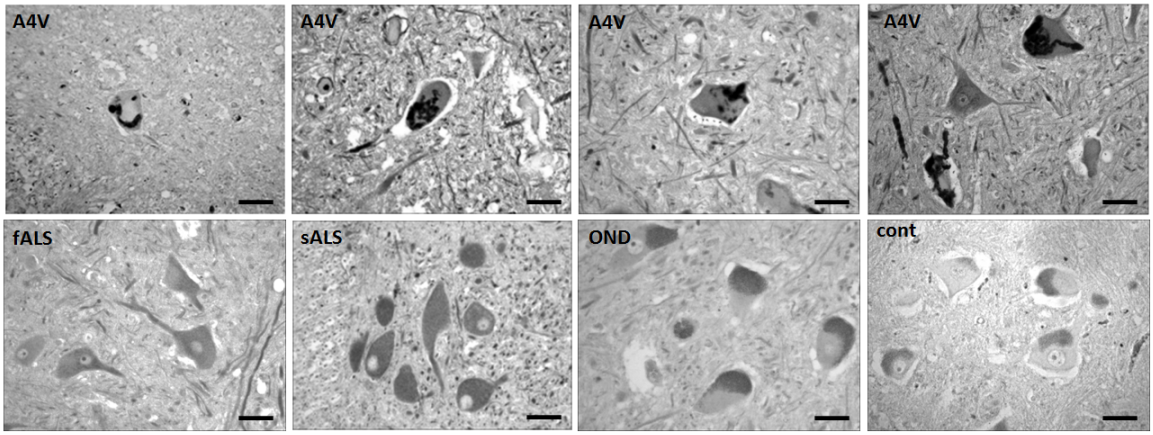

Figure 3.6: Mutant SOD1 human spinal cords show distinct skein-like intracellular inclusions immunoreactive with C4F6. All $SOD1^{A4V}$ cases examined (top row) show skein-like inclusions in motor neurons. Non-SOD1 fALS, sporadic ALS, other neurological diseases, and non-diseased controls (bottom row) show diffuse staining and do not exhibit skein-like inclusions. Scale bar = 50 μm.

	None	Sparse Diffuse	Majority Diffuse	Skein-like
A4V	0	0	0	3
fALS non-SOD1	0	0	2	0
sALS	0	10	15	0
OND	1	4	13	0
Non-diseased controls	1	4	0	0

Table 3.1. C4F6 immunoreactivity with human autopsy tissues.

3.4: Discussion

The identification of a subset of 'toxic' protein in fALS would be of considerable value to mechanistic research into the pathogenesis of ALS, potentially providing a target for development of therapeutics and screening for disease. Here we show that the C4F6 monoclonal antibody generated against mutant human SOD1 not only can differentiate between mutant and wild-type SOD1 protein, but also specifically reacts with mutant SOD1 protein present in tissues and cells affected by the disease. While our data do not directly demonstrate that the C4F6 immunoreactive protein is 'toxic', its propensity to localize only to the pathologically affected neuronal populations both in humans and rodent ALS models strongly suggests that this form of the mutant SOD1 protein plays a role in ALS pathogenesis. The differential reactivity of SOD1 proteins to C4F6 suggests structural variations in the proteins that could provide clues to the difficult question of why mutant SOD1 is preferentially toxic to motor neurons. Additionally, clinicians, pathologists, and patients have long been puzzled by the propensity of ALS to affect some motor neurons while seemingly leaving neighboring neuronal populations intact. Determining why C4F6 reacts only with a subset of neurons harboring mutant SOD1 proteins may help to solve this conundrum.

We approached the characterization of the SOD1 protein that reacts with C4F6 using a number of *in vitro* and *in vivo* methods. C4F6 immunohistochemistry and native immunoblot analysis distinguished the presence of mutant SOD1 proteins in diseased tissues (i.e. motor neurons) from clinically and pathologically unaffected tissues (e.g. sensory

neurons), suggesting the existence of different conformational epitopes of mutant hSOD1. In SDS-PAGE, C4F6 again reacted with mutant (but not wild-type) SOD1 protein in pathologically affected tissue; however, unaffected mutant tissues such as sensory neurons in the dorsal spinal cord were now recognized by C4F6 as well. Since proteins separated by SDS-PAGE are denatured and linearized, this finding suggests that the formation of a novel epitope through protein misfolding cannot fully account for C4F6 immunoreactivity. However, the linearized protein also is unlikely to be the sole immunoreactive epitope for C4F6. This conclusion is based on the finding that C4F6 discriminated mutant SOD1 from wild-type SOD1 in the linearized form. The possibility that immunoreactivity is based on the specific G93A point mutation is contradicted by the finding that C4F6 recognizes other linearized mutant SOD1 proteins (Urushitani et al., 2007). The precise C4F6 epitope has remained elusive, although epitope mapping has identified exon 4 (Bosco et al., 2010) as necessary to C4F6 immunoreactivity. Regardless, C4F6 is a valuable tool for tracking disease because it is able to discriminate mutant SOD1 protein that is present in pathologically affected versus non-affected tissues.

To further investigate whether C4F6 immunoreactivity is dependent on misfolding of mutant SOD1 protein, we used hydrophobic interaction chromatography to separate hydrophobic (misfolded) SOD1 from natively folded protein. Surprisingly, although we identified a fraction of mutant SOD1 protein that was hydrophobic, this fraction was only weakly C4F6 immunoreactive, whereas the non-hydrophobic fraction was robustly C4F6 immunoreactive. This does not rule out the possibility that C4F6 recognizes a misfolded hSOD1 species, but it does suggest that hydrophobicity is not a necessary characteristic of

this species. It is not clear why C4F6 does not robustly react with hydrophobic SOD1 once it has been denatured. As it is unlikely that the C4F6 epitope is either strictly linear or strictly conformational, there may be an additional modification necessary for C4F6 immunoreactivity that is not present at high levels in the hydrophobic fraction. An alternate explanation is that C4F6 is not sensitive enough to detect the relatively low levels of hydrophobic hSOD1; however, sensitivity assays suggested that this is not the case (figure 3.S4). Even so, protein not trapped by the column was C4F6-immunoreactive, demonstrating that hydrophobicity is not exclusively necessary for immunoreactivity.

It is clear that mutant SOD1 proteins are toxic to motor neurons, and there is an ongoing debate about whether this toxicity is due to the presence of SOD1 aggregates (Kopito, 2000; Rakhit et al., 2002; Rakhit et al., 2004; Matsumoto et al., 2005; Stojanovic et al., 2005; Basso et al., 2006; Chakrabartty et al., 2007; Basso et al., 2009; Witan et al., 2009). We found that the subset of mutant SOD1 protein that was recognized by C4F6 also was highly soluble, and that the less soluble mutant proteins did not react with the C4F6 antibody. This finding is consistent with previous data supporting the hypothesis that the toxic form of mutant SOD1 may not be wholly contained within protein aggregates (Zetterstrom et al., 2007; Witan et al., 2008; Witan et al., 2009; Prudencio et al., 2010). Indeed, data from models of Alzheimer and Huntington diseases suggest that by sequestering soluble, toxic forms of the pathogenic proteins, aggregation into larger polymers may actually serve a protective function (Bodner et al., 2006; Cohen et al., 2006; Outeiro et al., 2007).

To evaluate the clinical relevance of our rodent and *in vitro* data, we performed immunohistochemistry with the C4F6 antibody on autopsy tissues from ALS patients and controls. Patients carrying the A4V SOD1 mutation showed C4F6-immunoreactive skein-like intracellular inclusions within spinal motor neurons, similar to those seen in the rodent models. As in the rodent tissues, staining was restricted to the ventral horn, and only to a subset of motor neurons. This pattern of staining was not seen in non-SOD1 familial ALS, sporadic ALS, or in control tissue. C4F6 did stain some motor neuron populations in the non-SOD1 tissue but this staining was more diffuse, and was easily distinguished from the patterns seen in the SOD1 cases. Interestingly, this diffuse staining was seen not only in ALS, but also in motor neurons from patients dying with other neurological diseases and in non-neurological controls. Several possibilities could account for this; it may be that 1) other neurological diseases and/or stressors result in SOD1 becoming C4F6-immunoreactive; 2) C4F6 could be a more general marker of 'sick' neurons; or 3) the diffuse staining noted in sALS and neurological controls could be non-specific background and/or lipofuscin. Methodological issues cannot account for the pathological distinction between the ALS cases with and without SOD1 mutations. Varying antibody concentrations and incubation conditions and the use (or not) of antigen-retrieval techniques did not alter the conclusion that the SOD1 ALS patients show specific staining with C4F6. The finding of C4F6 diffusely stained motor neurons in sporadic ALS is consistent with the results reported by Bosco et al (Bosco et al., 2010). However, we also saw similar staining in non-ALS tissues; methodological differences between the laboratories may account for this discrepancy.

In summary, these data add to a growing body of literature investigating the specific properties of mutant proteins implicated in neurodegenerative diseases. We demonstrate that C4F6 is specific for pathologically affected tissue in humans and rodents expressing mutant SOD1, and believe it to be a marker of toxic SOD1 protein. The immunoreactive epitope is present primarily in the soluble protein fraction, and is specific neither for the G93A mutation nor for the exposed hydrophobic characteristic of a misfolded protein. The ability of C4F6 to detect a ubiquitously expressed protein only in pathologically affected tissue provides a novel means to investigate how and why motor neurons are uniquely targeted in this disease.

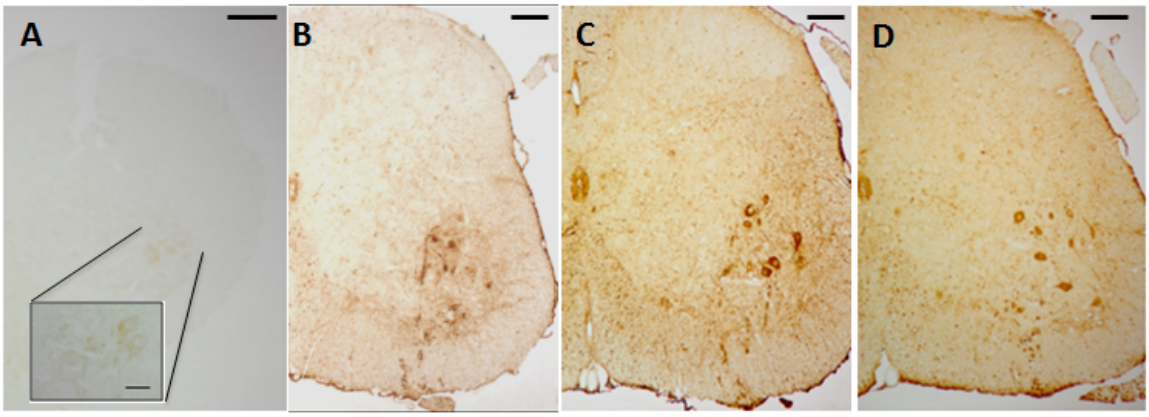

Figure 3.S1: C4F6 staining increases with age but maintains specificity for pathologically affected tissue. Spinal cord sections from 15 (A), 47 (B), 80 (C), and 120 (D) day $hSOD1^{G93A}$ mice. Note that C4F6 staining is quite faint at 15 days of age, and is present only in motor neurons (insert). At 47 days the immunoreactivity is easily recognized, and is present only in the ventral horn. More diffuse staining is seen at 80 and 120 days, but the majority of staining remains in the ventral horn and in the motor neurons. Scale bar = 100 μm (A, inset = 20 μm) or 200 μm (B-D).

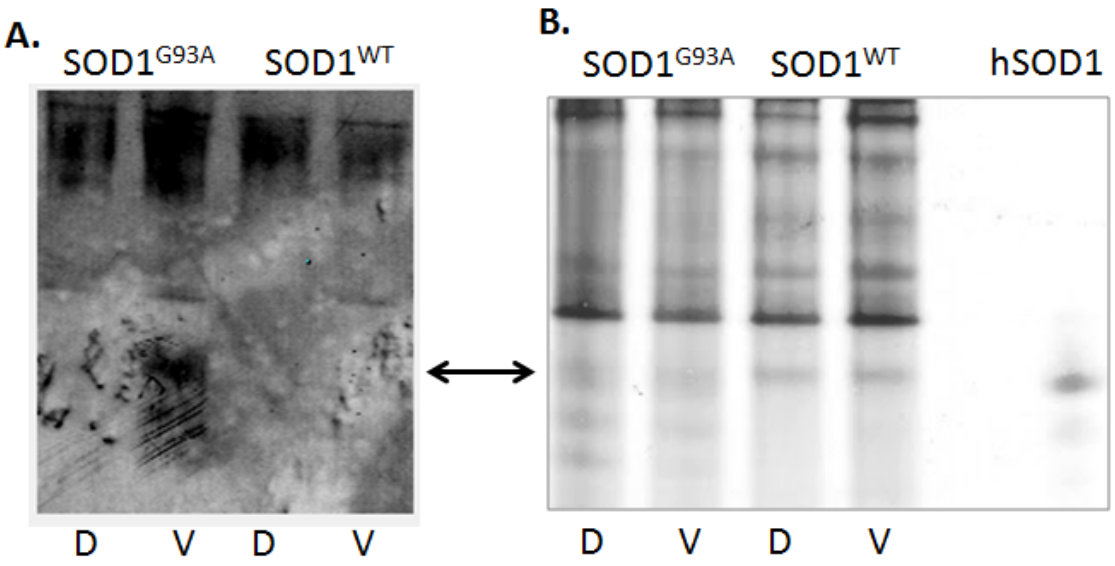

Figure 3.S2: C4F6 only detects non-denatured mutant hSOD1 from pathologically affected tissue. (A) Dorsal and ventral spinal cord homogenates (30 μg total protein) from hSOD1^{G93A} and hSOD1^{WT} animals were separated on a native, non-denaturing gel, transferred to PVDF membranes, and probed with the C4F6 antibody. C4F6 did not react with non-denatured mutant hSOD1 from the dorsal spinal cord, but did react with non-denatured mutant hSOD1 from ventral cord. C4F6 did not react with non-denatured wild-type hSOD1 from dorsal or ventral spinal cord. C4F6 reacted non-specifically with protein corresponding to the position of the stacking gel in all samples. (B) Coomassie protein detection on a native gel run in parallel confirms that pure hSOD1 (2 μg) migrates to the same position as the C4F6 immunoreactive smear specific to the hSOD1^{G93A} ventral spinal cord (double-headed arrow), providing evidence that this smear is human hSOD1.

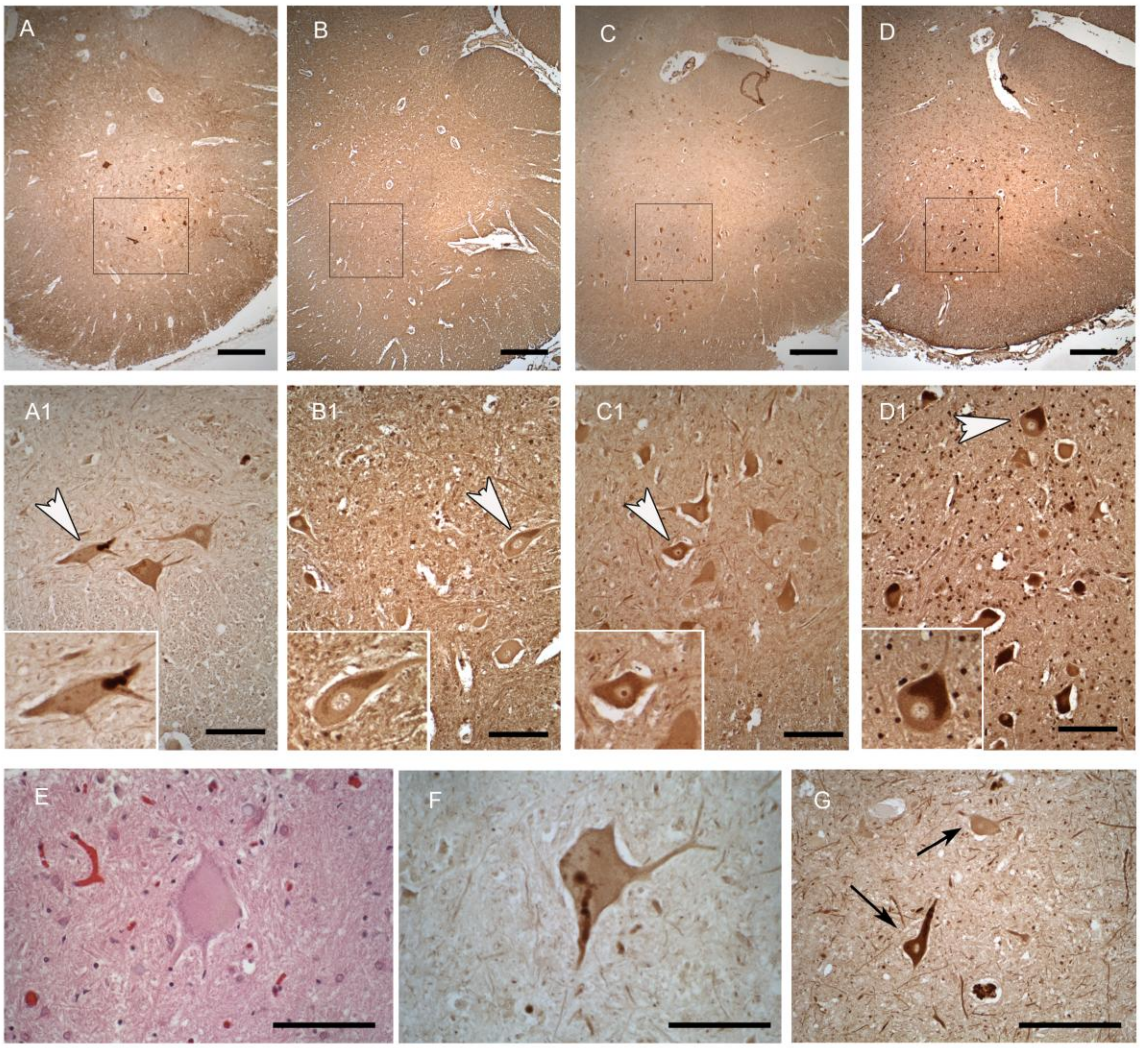
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Figure 3.S3: Replacement of C4F6 with normal mouse serum shows diffuse, but not skein-like staining in human spinal cord. Ventral horn in A4V fALS (A,B) or sALS (C, D) spinal cord stained using C4F6 (A,C) or normal mouse serum (B,D) as the primary antibody. A1-D1 are high powered pictures of boxed regions in A-D, and insets provide higher power images of neurons identified with the arrowheads, which are representative of motor neurons in these sections. Note the focal, globular staining in A4V tissue stained with C4F6 (A1), as opposed to diffuse staining in sALS tissue stained with C4F6 (C1). Staining of A4V (B1) and sALS (D1) tissue replacing C4F6 with normal mouse serum showed diffuse staining. In addition, many motor neurons from all patients (ALS and control) showed the presence of lipofuscin, which stains non-specifically with normal mouse serum (D1, arrowhead). Panel E shows a chromatolytic motor neuron in an A4V spinal cord. This same motor neuron (seen in a serial section) contains a C4F6 immunoreactive inclusion (F), supporting the hypothesis that C4F6 identifies mutant SOD1 protein in degenerating neurons. Panel G shows adjacent neurons (arrows) in the spinal cord of an $SOD1^{A4V}$ patient that are differentially stained with the C4F6 antibody, similar to the pattern seen in mutant SOD1 rodents (figure 1). Scale bars= 500 μm A-D = 100 μm all others.

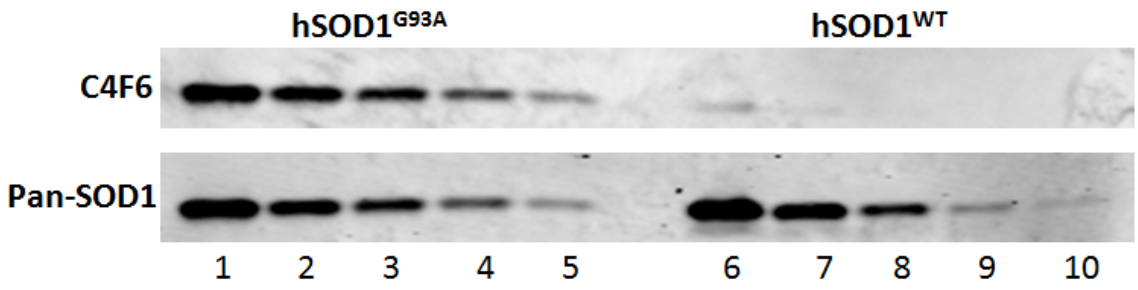


Figure 3.S4: C4F6 detects very low levels of denatured SOD1^{G93A} protein. Protein homogenates from hSOD1^{G93A} and hSOD1^{WT} mouse spinal cords were serially diluted, separated on SDS-PAGE and probed with C4F6 (top) and a pan-SOD1 antibody (bottom) to evaluate C4F6 sensitivity.

Lanes 1-5 are hSOD1^{G93A} (lane 1: 1.25,2: 0.68,3:0.34,4: 0.17,5: 0.08 μg total protein) and lanes 6-10 are hSOD1^{WT} (lane 6: 20, 7:10,8:5,9: 2.5,10:1.25 μg total protein). C4F6 detects the hSOD1^{G93A} protein at a level of 0.08 μg total protein. Note that C4F6 is weakly immunoreactive with hSOD1^{WT} when high levels (20 μg) of protein are present.

Chapter 4

Cellular toxicity of mutant SOD1 protein is linked to an easily soluble, non-aggregated form *in vitro*

Abstract

Mutations in superoxide dismutase 1 (SOD1) are found in approximately 20% of patients with familial amyotrophic lateral sclerosis. The propensity of mutant SOD1 to form aggregates in pathologically affected cells (i.e. motor neurons) has implicated these poorly soluble protein aggregates and/or their misfolded soluble precursors as being instrumental to the disease process. We investigated the relative solubility and toxicity of four different mutant SOD1 proteins in a cell-based model system and demonstrate that the mutant, misfolded SOD1 proteins that are the most soluble are also the most toxic. This toxicity was ameliorated by upregulating heat-shock protein chaperones in order to refold the soluble, misfolded protein, regardless of the presence of poorly soluble SOD1. We further demonstrate that increasing the solubility of a SOD1 mutant protein that is both poorly soluble and non-toxic, as compared to other mutant proteins, resulted in remarkably increased toxicity of the mutant SOD1. Again, this increased toxicity was attenuated by upregulating heat-shock protein chaperones in order to refold the soluble, misfolded proteins. These findings implicate easily soluble, misfolded SOD1 as being toxic to the cell and support the hypothesis that reducing solubility of mutant SOD1 proteins through aggregation may occur as a self-protective response in the cell.

4.1: Introduction

Protein aggregation is a pathological hallmark of many neurodegenerative diseases, including ALS (Ross and Poirier, 2004). SOD1 is a primary component of protein aggregates found in patients carrying SOD1 mutations and in transgenic mutant SOD1 rodent disease models. Antibodies that are specific for misfolded forms of the SOD1 protein have also shown that granular SOD1 inclusions can be found in autopsy tissues from people with sporadic ALS, providing a possible mechanistic link between the sporadic and familial forms of disease (Bosco et al., 2010; Forsberg et al., 2010). The role of these aggregates in disease pathogenesis is unclear. Past research has focused heavily on aggregates being toxic to the cell, but recent evidence implies that the aggregates may actually be innocuous, or even play a cytoprotective role (Walsh et al., 2002; Finkbeiner et al., 2004; Bodner et al., 2006). Mutant SOD1 is known to misfold into a disordered structure, and it may be that it is this soluble, misfolded form of SOD1 that is responsible for cellular toxicity (Shaw et al., 2006; Durazo et al., 2009). In this paradigm, aggregates are formed as a compensatory response in order to sequester toxic, improperly folded soluble protein. In support of this theory are data from transgenic mutant SOD1 rodents demonstrating that soluble, misfolded SOD1 is enriched in pathologically affected tissues prior to disease onset, whereas SOD1 aggregates increase most markedly after symptom onset (Johnston et al., 2000; Wang et al., 2002a; Jonsson et al., 2004; Zetterstrom et al., 2007). The relative dearth of SOD1 aggregates pre-symptomatically implies that SOD1 aggregates may not be the primary driver of disease initiation. In contrast, because improperly folded soluble SOD1 *is* present prior to symptom onset and accumulates in pathologically affected tissue leading up to symptom onset, the culpable, toxic, SOD1

species may be a soluble and non-aggregated form (Zetterstrom et al., 2007; Wang et al., 2009a).

We examined four disease-causing human SOD1 (hSOD1) mutants – A4V, G37R, G93A, and G93C- and the wild-type hSOD1 protein, and compared their relative solubility with their propensity to initiate cell death. Our data demonstrate that the degree of cellular toxicity consistently correlates with the amount of easily soluble, but misfolded mutant SOD1 protein. Indeed, for each of the mutants, refolding of misfolded soluble mutant protein reduced toxicity, and increasing the level of soluble mutant protein increased toxicity. These data support the hypothesis that a readily soluble, non-aggregated form of mutant SOD1 may be an important mediator of mutant SOD1-mediated toxicity.

4.2: Materials and Methods

Cell Culture

Chinese Hamster Ovary (CHO) cells were maintained in Gibco F-12 Hams media supplemented with fetal bovine serum, L-glutamine and penicillin/streptomycin. Cells were transiently transfected at 95% confluency with Lipofectamine 2000 according to manufacturer protocols. In co-transfections, the ratio of wild-type: mutant hSOD1 DNA was 1:1, and the total amount of hSOD1 DNA transfected in co-transfection or mutant only conditions was constant.

Pharmacological administration

Cells were treated with 2.5 μM MG-132 (Calbiochem), 10 μM geldanamycin (Alomone Labs), or DMSO vehicle 24 hours after transfection. Drug treatment protocols were as follows: 24 hour treatment with MG-132, geldanamycin, or vehicle, 48 hour treatment with MG-132 alone, 48 hour treatment with MG-132 with geldanamycin also being present for the last 24 of those 48 hours, or 48 hour treatment with DMSO vehicle.

Cytotoxicity

Cytotoxicity was evaluated using Invitrogen's Live/Dead Cytotoxicity Kit for Mammalian Cells (L-3224) according to manufacturer protocols. All viability experiments were repeated three times; a minimum of 100 cells were counted per condition during each trial. Cell counts were obtained using the automated cell counting software Image-Pro Plus 5.1.

Solubility Fractionation

Cells were homogenized through 3 cycles of freeze/thaw on dry ice in phosphate-buffered saline in the presence of protease inhibitors (Roche, Mini Complete cocktail tablets) and then centrifuged at 16,000 x *g* for 30 minutes at 4°C. The supernatant was removed as the "readily soluble" fraction, and the pellet was washed 3 times in PBS prior to suspension in a HEPES-based buffer containing 1.5% NP-40, 2% sodium dodecyl sulfate (SDS), and 0.25% deoxycholic acid and protease inhibitors. The homogenate was sonicated 3 x 5 seconds; the resulting suspension contained the "detergent-soluble" protein fraction. Uniform amounts of total protein were separated through SDS-PAGE on a 4-20% gradient gel and transferred to PVDF membrane through semi-dry transfer. Membranes were blocked for 1 hour in 5% filtered non-fat milk prior to overnight incubation with primary antibody at 4°C

(Calbiochem pan-SOD1 sheep polyclonal 1:1000, Sigma-Aldrich β-actin mouse monoclonal, 1:5,000, Millipore ubiquitin mouse monocolonal 1:1,000, or EnzoLife Science Hsp70/Hsp72 mouse monoclonal, 1:100). The next morning, membranes were washed in PBS/Tween before 1 hour incubation in near-infrared (IR) dye conjugated secondary antibody (Rockland) at room temperature. The membrane was washed extensively and visualized on an Odyssey (Li-Cor Biosciences) scanner. Optical densitometry (OD) values for hSOD1 bands were obtained with NIH ImageJ software. The percent "readily soluble" protein was defined as: readily soluble hSOD1 OD/(readily + detergent-soluble OD)*100 for each mutant. Experiments were performed and quantified 3 times.

Proteasome Activity

CHO cells were treated for 24 hours with 2.5 μM MG-132 or vehicle, activity was evaluated with Millipore Proteasome Activity Assay (Cat No APT280) according to manufacturer protocols.

Heat Shock

CHO cells were placed in a 42°C incubator for 1 hour (or maintained at 37°C) before being returned to a 37°C incubator overnight for recovery. Cells that were both treated with MG-132 and heat-shocked were treated with 2.5 μM MG-132 directly before heat shock.

Statistical Analysis

Statistical analysis (GraphPad InStat 3 software) was based on at least 3 independent experiments. For comparisons of wild-type and mutant protein solubility and toxicity, we performed one-way ANOVA with a post-hoc Tukey's test (figures 4.2B and 4.2C). The same

statistical analysis was used for the pharmacological treatments for each mutant in figures 4.4, 4.6, 4.S4 and 4.S5. Unpaired t-tests compared differences between drug and vehicle treatments in figures 4.3 and 4.5D, between mut-mut and WTFLAG-mut for figures 4.5B, 4.5C, and 4.S7, between heat shock conditions in figure 4.S6, and for proteasome activity in figure 4.S2.

4.3: Results

In this study, we present data correlating toxicity and solubility of four different hSOD1 mutants in Chinese Hamster Ovary (CHO) cells. We chose CHO cells because they are strongly adherent and because they do not endogenously express human SOD1. It is important to use a model system that does not endogenously express hSOD1 in order to ensure that dimers consisting of the endogenous and transiently expressed SOD1 do not form. Additionally, rodent and human SOD1 run at different molecular weights, so in this model, endogenous and transiently transfected SOD1 can be differentiated in electrophoretic gels. Past studies indicate that mutant SOD1 pathology is conserved in CHO cells (Stieber et al., 2004; Prudencio and Borchelt, 2011). The mutants chosen were selected because they are well-characterized and are scattered throughout the three-dimensional structure of the SOD1 homodimer (figure 4.1). A4V, the most common SOD1 mutation among ALS patients in North America, is associated with a rapidly progressive form of disease (Rosen et al., 1994) and is located at the dimer interface. G37R and G93A hSOD1 mice are well-characterized rodent models of disease; G37R is located within the SOD1 β-

barrel and G93A is located in the solvent-exposed β-barrel plug. The G93C mutation represents a variation on G93A.

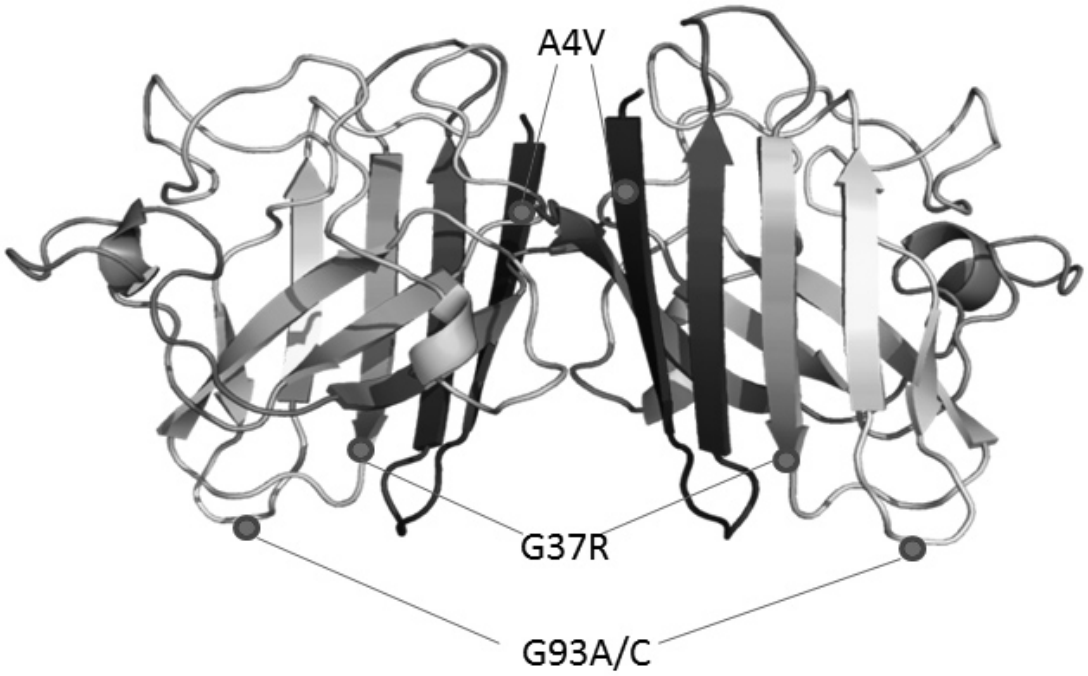
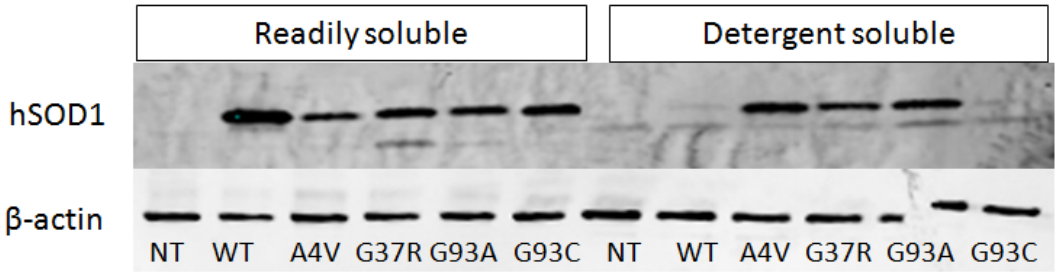


Figure 4.1: Localization of SOD1 mutants in wild-type structure. A4V, G37R, G93A, and G93C are identified in the hSOD1 homodimer. Structure modified from Protein Data Bank, PDB 2C9V (Strange et al., 2006).

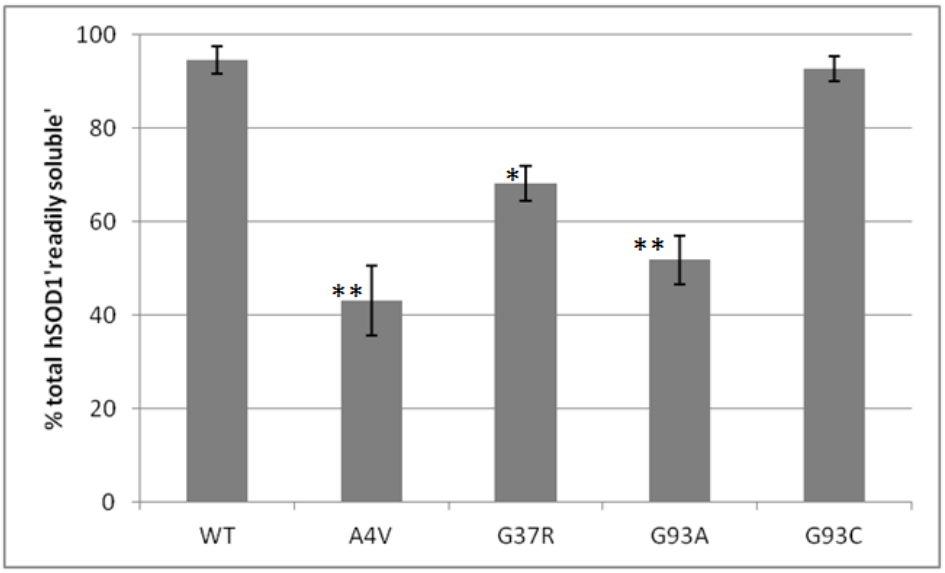
Solubility differs among hSOD1 mutants

The mutant and wild-type hSOD1 proteins did not demonstrate uniform solubility. hSOD1 transgenes were transiently expressed in CHO cells for 48 hours prior to harvesting. Similar levels of protein expression were demonstrated for each of the mutant hSOD1 proteins (figure 4.S1). Cellular homogenates were separated into two components: 1) a "readily soluble" fraction harvested without the addition of exogenous solubilizing agents, and 2) a "detergent-soluble" fraction (1.5% NP-40, 2% SDS, and 0.25% deoxycholic acid) that contained the majority of remaining cellular proteins. This strategy was used to isolate the SOD1 protein that is inherently soluble within the cytosol from the SOD1 protein that is less soluble, and potentially contained within some aggregated form. The solubility fractions were subjected to immunoblot analysis, probed with a pan-SOD1 antibody, and the relative amounts of SOD1 in each fraction were quantified through densitometry. For WT and G93C hSOD1, the majority of SOD1 protein was found in the readily soluble fraction, whereas a significant proportion of SOD1 protein was found in the detergent-soluble fraction for A4V, G37R, and G93A (figure 4.2A, B). The "least soluble" mutant, that is, the mutant that had the greatest proportion of SOD1 in the detergent-soluble fraction, was A4V.

A.


Readily soluble
Detergent soluble
hSOD1
β-actin
NT WT A4V G37R G93A G93C NT WT A4V G37R G93A G93C

B.


% total hSOD1 'readily soluble'
100
80
60
40
20
0
**
*
**
WT
A4V
G37R
G93A
G93C

C.

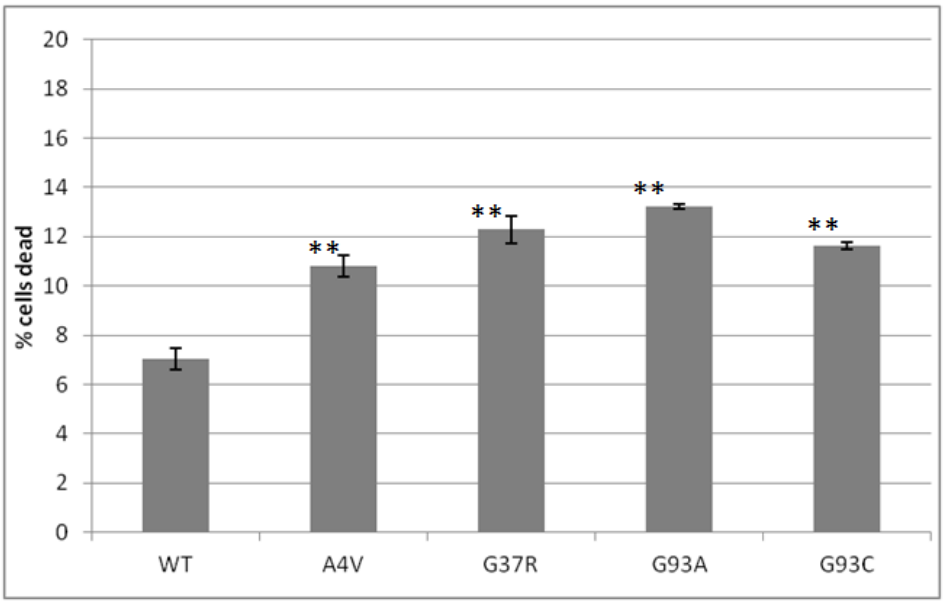

% cells dead
20
18
16
14
12
10
8
6
4
2
0
**
**
**
**
WT
A4V
G37R
G93A
G93C

Figure 4.2: Solubility, but not toxicity, differs across non-stressed hSOD1 mutants. A) CHO cells transiently expressing WT, A4V, G37R, G93A, or G93C hSOD1 for 48 hours were separated into readily and detergent-soluble fractions and detected through immunoblot using a pan-SOD1 antibody. β-actin levels demonstrate equal protein loading. NT indicates non-transfected cells. B) Levels of readily and detergent-soluble hSOD1 were quantified by optical densitometry; the percent readily soluble hSOD1 was expressed over total hSOD1 for each mutant. WT and G93C protein were the most soluble, A4V was the least soluble. C) 48 hours following transient transfection, viability was assessed through membrane integrity. The mutants did not differ from each other in terms of toxicity; however, all mutants exhibited a weak toxicity compared to wild-type hSOD1. Values represent the means ± SEM, and significance is relative to the wild-type hSOD1 condition (* = $p<0.05$, ** = $p<0.001$).

The effect of the expression of each of the mutant proteins on cell viability was evaluated using Invitrogen's cell viability assay, as described under Methods. In this assay, live cells are identified by intracellular esterase activity, and dead cells are detected through loss of plasma membrane integrity. All of the cells expressing mutant SOD1 proteins showed weak toxicity when compared to cells expressing the wild-type hSOD1 protein; however, there were no differences in cellular toxicity among the four mutants (figure 4.2C).

An increase in soluble mutant hSOD1 correlates with toxicity

In order to investigate the relative toxicities of mutant hSOD1 proteins in our model, we needed to stress the cells to induce significant levels of cell death. We chose proteasome inhibition as the stressor because the primary function of the proteasome is protein turnover, and misfolded and/or damaged proteins are degraded through the ubiquitin-proteasome pathway (Hershko and Ciechanover, 1982, 1986). Therefore, inhibition of the proteasome is expected to increase levels of both soluble misfolded, and aggregated proteins. Additionally, proteasome activity in the lumbar spinal cord of G93A mice is decreased throughout disease, making this a relevant form of cellular stress (Kabashi et al., 2004). When hSOD1-expressing cells were exposed to the proteasome inhibitor MG-132 for 24 hours, we found a corresponding increase in levels of hSOD1 in both readily soluble (figure 4.3A) and detergent-soluble fractions. Notably, whereas the amount of hSOD1 present in each of these fractions increased in the presence of MG-132, the relative proportions of hSOD1 found in the different fractions did not differ from the control condition. Proteasome inhibition was verified using both a proteasome activity assay and estimation of relative levels of polyubiquitinated proteins (figure 4.S2).

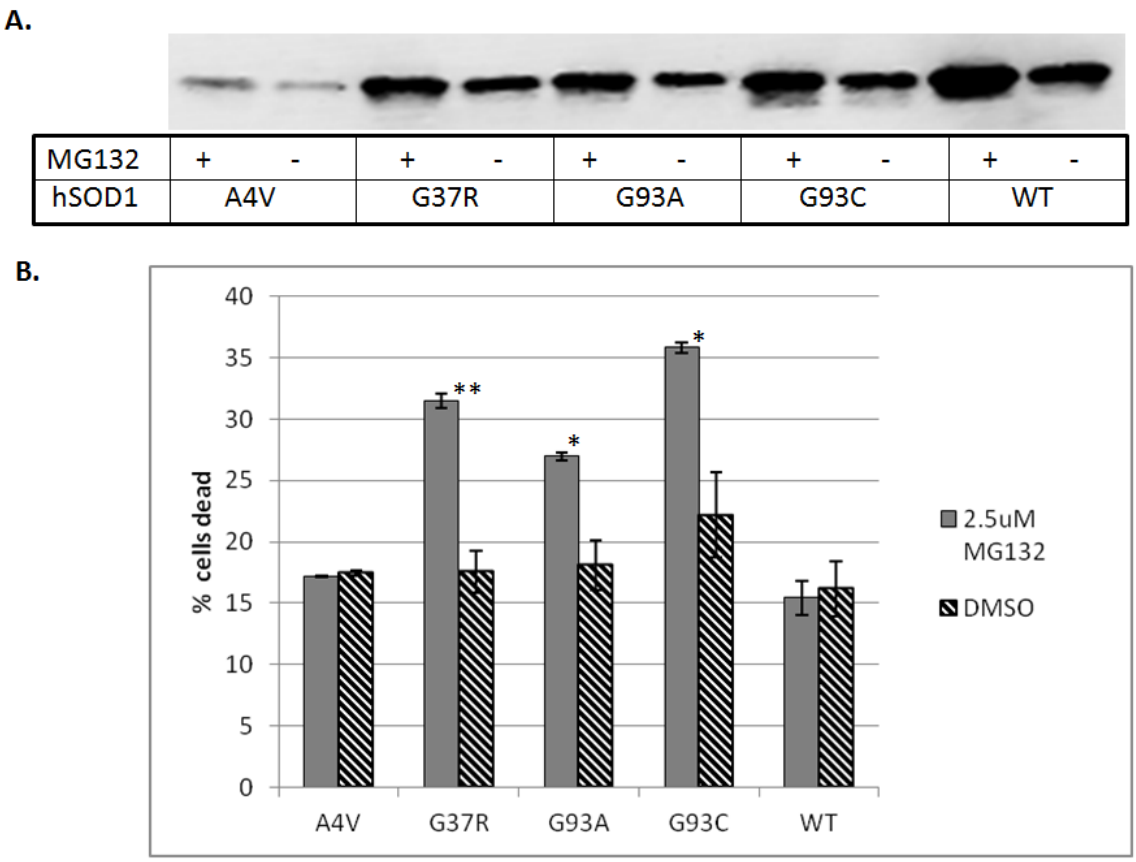


Figure 4.3: SOD1-related toxicity correlates directly with relative solubility. A) 24 hours after SOD transfection, CHO cells were exposed to MG-132 (2.5 μM) for 24 hours. The homogenates were separated into readily soluble and detergent-soluble fractions and then detected through immunoblot using a pan-SOD1 antibody. The immunoblots demonstrate that the levels of readily soluble protein increase in the presence of MG-132. B) Proteasome inhibition differentially affects SOD1-related toxicity in cells expressing the different hSOD1 mutants. Values represent the means of 3 independent experiments, ± SEM. Significance is based on comparison of means between MG-132 and DMSO treatments (* = $p<0.05$, ** = $p<0.005$).

As predicted, proteasome inhibition produced significant toxicity in cells expressing mutant hSOD1. Importantly, no changes in toxicity were detected in cells expressing WT hSOD1. The degree of cellular toxicity varied among the cells expressing the four different mutant proteins (figure 4.3B), and relative toxicity correlated directly with the relative solubility of each mutant protein (figure 4.2B). Specifically, the cells expressing G93C, which showed the highest proportion of readily soluble protein, exhibited the greatest increase in toxicity when exposed to MG-132 for 24 hours, whereas cells expressing A4V, the least soluble among the mutant proteins, showed very little increase in toxicity with proteasome inhibition. Cells expressing G37R and G93A showed intermediate toxicities relative to A4V and G93C. The strong correlation between solubility and toxicity across the hSOD1 mutants suggests that the toxic hSOD1 species is found in the readily soluble fraction. These data also support the idea that the soluble, toxic SOD1 protein is likely misfolded, since the proteasome is responsible for degradation of misfolded proteins and proteasome inhibition increased toxicity in cells expressing mutant, but not wild-type, hSOD1 protein. However, because proteasome inhibition increased levels of the both overexpressed wild-type and mutant hSOD1, it was necessary to further verify that the accumulating mutant protein was indeed misfolded.

To test the hypothesis that the toxic, soluble SOD1 proteins were misfolded, we sought to promote their refolding, predicting that this would reduce toxicity. To refold the soluble, misfolded SOD1 protein we induced transcription of heat shock chaperones, including heat shock protein 70 (Hsp70). Hsp70 is instrumental to protein quality control and is known to act in concert with other heat shock co-chaperones to assist in nascent protein folding, as well as refolding of soluble misfolded proteins, and targeting of proteins

for degradation (Beckmann et al., 1990; Rudiger et al., 1997; Batulan et al., 2006). Previous studies of aggregate-related toxicity could not distinguish between the effects of SOD1 aggregates and their misfolded, readily soluble precursors because aggregates are formed from these precursors, and so reducing the levels of misfolded, soluble protein would necessarily also reduce the presence of aggregates. To circumvent this issue, we designed an experiment in which we could separate the toxic effects of readily soluble versus detergent-soluble SOD1 protein. We used proteasome inhibition to increase the levels of both pools and then selectively refolded the soluble, misfolded fraction by upregulating heat shock chaperones. Because aggregate formation mediated by proteasome inhibition is a reversible process (Puttaparthi et al., 2003), we maintained proteasome inhibition throughout the experiment. This paradigm allowed us to observe the toxic effects of the detergent-soluble SOD1 in the absence of the misfolded, readily soluble precursors.

Heat shock chaperones were induced pharmacologically with the Hsp90 inhibitor geldanamycin, and increased Hsp70 levels were confirmed by immunoblot (figure 4.S3). In the absence of cell stress, Hsp90 complexes with heat shock transcription factor 1 (Hsf-1) and holds it in an inactive state, thereby preventing the induction of Hsp70 and other co-chaperones. Inhibiting Hsp90 with geldanamycin releases Hsf-1 from this complex, allowing Hsf-1 to induce transcription of heat shock chaperones, including Hsp70 (Shen et al., 2005). So, in this paradigm, cells are first stressed by exposure to the proteasome inhibitor MG-132 for 24 hours to induce the accumulation of misfolded proteins, and then are exposed to both MG-132 and geldanamycin for the next 24 hours in order to up-regulate heat shock chaperones to specifically target refolding of the misfolded, readily soluble protein, while maintaining levels of the detergent-soluble protein. There are reports that the heat shock

chaperone Hsp70 may play a role in disaggregating already formed inclusions (Liberek et al., 2008). However, we found that up-regulating Hsp70 in the presence of an inhibited proteasome did not decrease levels of already accumulated "less soluble" protein in our system (figure 4.S4).

Using this experimental paradigm, we found that up-regulating heat shock chaperones with geldanamycin reduced the cellular toxicity initiated by proteasome inhibition (figure 4.4). As with our previous experiments using proteasome inhibition, the mutants exhibited different levels of toxicity in the presence of MG-132 at 24 hours, and this toxicity increased with continued exposure to MG-132 for the next 24 hours. However, when cells first were exposed to MG-132 for 24 hours and then subsequently exposed to both MG-132 and geldanamycin for the next 24 hours, the increased cellular toxicity of MG-132 at 48 hours was ameliorated. Similarly, when cells were exposed to both MG-132 and geldanamycin from the outset, geldanamycin prevented MG-132-mediated toxicity (figure 4.S5). In order to confirm the protective effect of heat shock chaperones in this model, we also induced expression of heat shock proteins using heat shock (42°C x 1 hour). This paradigm yielded the same results as treatment with geldanamycin (figure 4.S6). The convergence of geldanamycin treatment and heat shock in both up-regulating heat shock chaperones and ameliorating cell death supports the hypothesis that these induced chaperones are responsible for attenuating MG-132-induced toxicity.

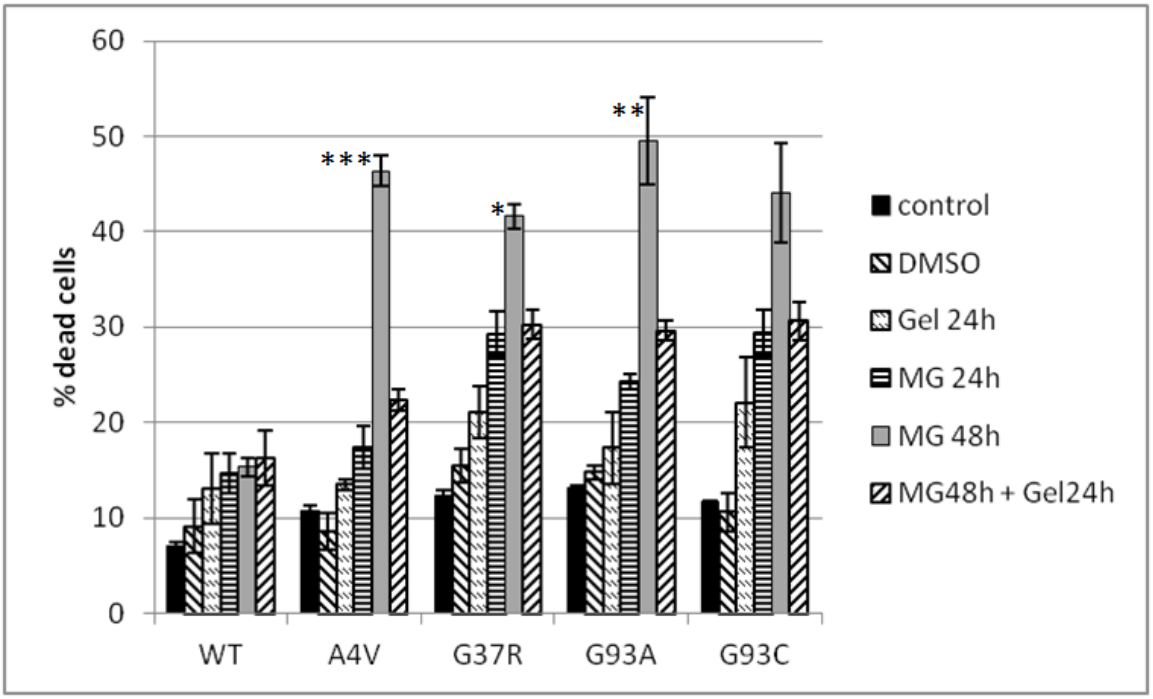


Figure 4.4: Upregulating Hsp70 ameliorates toxicity induced by proteasome inhibition. Transiently transfected CHO cells were treated at 24 hours post transfection with: DMSO (veh); 2.5 μM MG-132 for 24 hours; 10 μM geldanamycin (Gel) for 24 hours; 2.5 μM MG-132 for 48 hours; or 2.5 μM MG-132 for 48 hours with the cells simultaneously being exposed to 10 μM geldanamycin for the last 24 hours. Values represent the means of 3 independent experiments, ± SEM. Statistical analysis was based on comparisons of cell death in the MG48h condition vs. the MG48h + Gel24h condition for each mutant (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

The protective effect of up-regulating heat shock chaperones was not due to a decrease in mutant SOD1. Solubility profiles showed that the relative levels of readily and detergent-soluble proteins were conserved with pharmacologic manipulation (figure 4.S4). This suggests that the attenuation of toxicity with geldanamycin was not due to an overall reduction in levels of detergent-soluble protein or a transfer of the detergent-soluble protein to the readily soluble fraction. Indeed, inducing heat shock chaperones in mutant hSOD1-expressing cells attenuated MG-132-induced toxicity regardless of the presence of detergent-soluble SOD1, supporting the hypothesis that the toxic SOD1 species is not only misfolded, but is also very soluble. These data may seem to contradict the hypothesis that levels of soluble SOD1 correlate with toxicity. However, we interpret the data as showing that it is the SOD1 conformer within the soluble fraction that determines toxicity, and not simply levels of soluble SOD1. That is, the toxic SOD1 protein species is both readily soluble and misfolded. Refolding of the toxic misfolded protein with heat shock chaperones reduced toxicity, even though the total level of soluble SOD1 did not change.

Increasing mutant SOD1 solubility increases cellular toxicity

In the experiments up to this point, we had increased total levels of both readily soluble and detergent-soluble misfolded SOD1, showing a corresponding increase in toxicity in the mutants demonstrating the highest proportion of readily soluble protein. Targeted refolding of readily soluble protein by inducing heat shock chaperones ameliorated this toxicity, suggesting that the toxic form of the mutant SOD1 protein is both soluble and misfolded. To more directly address the question of how mutant SOD1 solubility impacts toxicity, we needed to increase the inherent solubility of the individual SOD1 mutants to

show that specifically increasing the proportion of mutant hSOD1 in the readily soluble fraction would also increase its toxicity. Past studies demonstrated that co-expressing mutant and wild-type hSOD1 increases mutant SOD1 solubility (Witan et al., 2008; Witan et al., 2009). Therefore, we transfected either equal amounts of wild-type and mutant hSOD1 DNA, or transfected double the amount of mutant hSOD1 DNA. In this manner, the total amount of hSOD1 DNA transfected was uniform regardless of whether the cells were expressing mutant only, or mutant plus wild-type protein. To differentiate co-expressed wild-type and mutant hSOD1 proteins, an N-terminal 3X-FLAG tag was added to the wild-type hSOD1.

As predicted, the co-expression of wild-type SOD1-FLAG and mutant hSOD1 increased the relative solubility of the mutant protein (figure 4.5A, B). The relative solubility of mutant hSOD1 was evaluated independently for the mut-mut and mut-WTFLAG conditions. The greatest increase in mutant SOD1 solubility in the presence of wild-type hSOD1, as compared to the mutant-mutant condition, was found in cells expressing A4V and G93A proteins. These were also the mutants that were the least soluble in the absence of wild-type protein and so had the greatest potential for increased solubility. We next evaluated the effect of co-expressing wild-type hSOD1 on toxicity; the presence of wild-type protein increased toxicity in cells expressing A4V, G37R, and G93A hSOD1 (figure 4.5C). The greatest increase in toxicity as compared to the mutant-mutant condition was found in A4V and G93A hSOD1-expressing cells. Notably, these are the same mutants that showed the greatest increase in solubility in the presence of the wild-type protein.

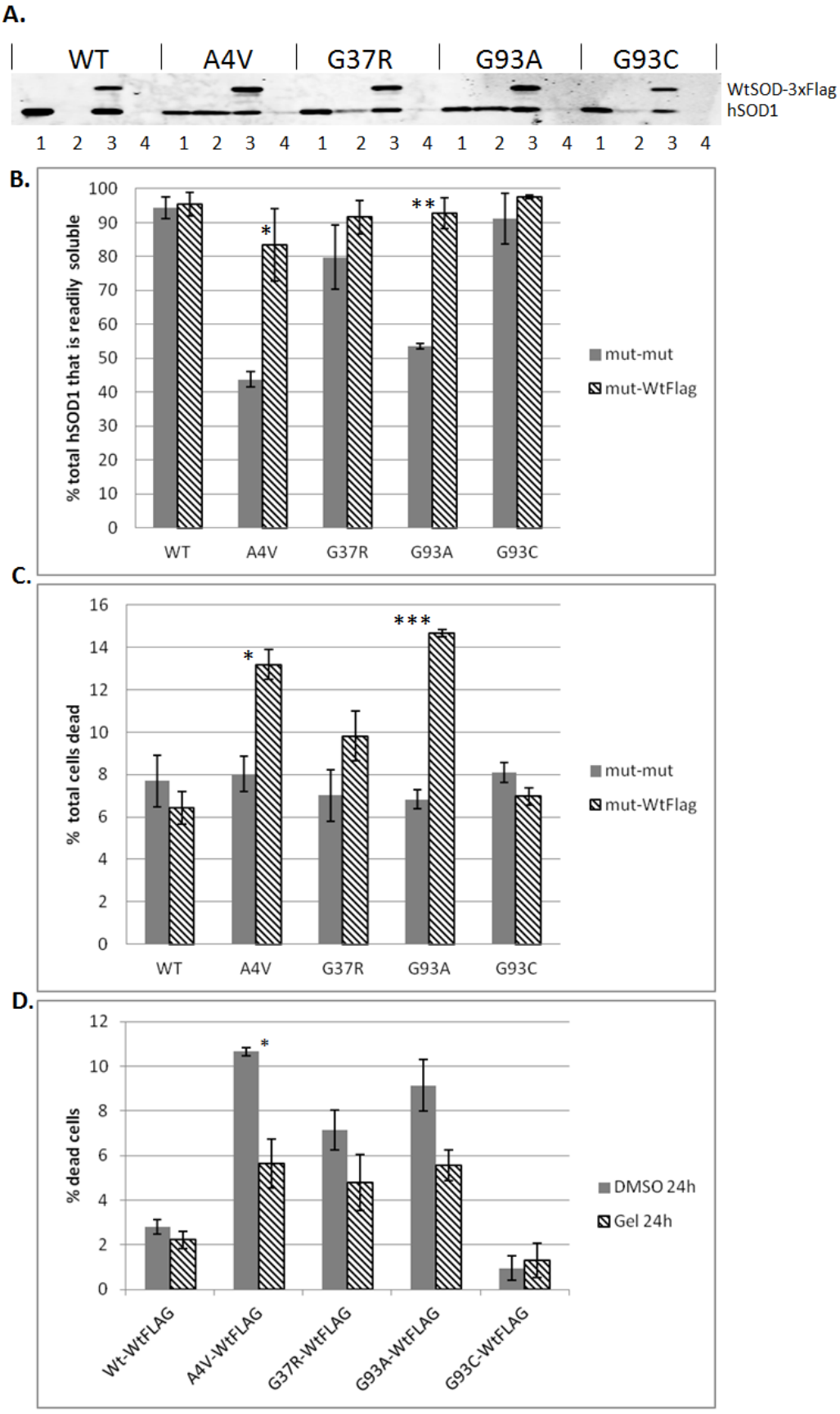

A.
WT
A4V
G37R
G93A
G93C
WtSOD-3xFlag
hSOD1
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
B.
% total hSOD1 that is readily soluble
100
90
80
70
60
50
40
30
20
10
0
*
**
WT
A4V
G37R
G93A
G93C
mut-mut
mut-WtFlag
C.
% total cells dead
16
14
12
10
8
6
4
2
0
*
***
WT
A4V
G37R
G93A
G93C
mut-mut
mut-WtFlag
D.
% dead cells
12
10
8
6
4
2
0
*
Wt-WtFLAG
A4V-WtFLAG
G37R-WtFLAG
G93A-WtFLAG
G93C-WtFLAG
DMSO 24h
Gel 24h

Figure 4.5: Wild-type hSOD1 increases both mutant hSOD1 solubility and toxicity; this increased toxicity can be rescued by upregulating Hsp70. A) CHO cells were transiently transfected with equal total amounts of mutant (mut-mut) or mutant and wild-type-3xFLAG (mut-WtFLAG) hSOD1 DNA; 48 hours later protein was separated into readily soluble and detergent-soluble fractions, and detected on immunoblot with a pan-SOD1 antibody. Lane (1)=readily soluble mut-mut, (2)=detergent-soluble mut-mut, (3)=readily soluble mut-WtFLAG, (4)=detergent-soluble mut-WtFLAG; the Wt-3xFLAG protein has a slightly higher molecular weight. B) Bands were quantified using optical densitometry, confirming that co-expression with WT hSOD1 increases the proportion of readily soluble mutant protein for A4V and G93A. C) The increased proportion of readily soluble mutant SOD1 protein corresponds with toxicity. WT hSOD1 induced toxicity in A4V, G37R, and G93A expressing cells relative to the mut-mut condition. D) Induction of heat shock proteins with geldanamycin (10 μM for 24 hours) protects against toxicity created by co-expression with WT hSOD1 protein. For B and C, statistical comparisons are mut-WtFLAG vs. the mut-mut condition. For D, statistical comparisons are with the DMSO condition for each hSOD1 mutant. Values represent the means of 3 independent experiments ± SEM (* = $p<0.05$, ** = $p<0.001$, *** = $p<0.0001$).

The increase in toxicity caused by co-expression of wild-type SOD1 was rescued by treatment with geldanamycin (figure 4.5D), again suggesting that the increased soluble fraction of mutSOD1 contains misfolded, toxic protein. Heat-shocking the cells at 42°C for one hour similarly rescued viability, supporting the hypothesis that geldanamycin mediates viability via the heat shock pathway (figure 4.S7). Additionally, increased solubility due to co-expression of wild-type and mutant hSOD1 also increased the SOD1-related toxicity in cells exposed to proteasome inhibition (figure 4.6). Again, A4V and G93A, the SOD1 mutants that exhibited the greatest increase in solubility when co-expressed with the wild-type protein, showed the greatest sensitivity to MG-132 when co-expressed with wild-type hSOD1. Notably, A4V-expressing cells were resistant to MG-132-induced toxicity in the absence of wild-type protein (figure 4.3), and the co-expression of wild-type hSOD1 with A4V transformed a comparatively non-toxic mutant into a toxic one, presumably by increasing its solubility.

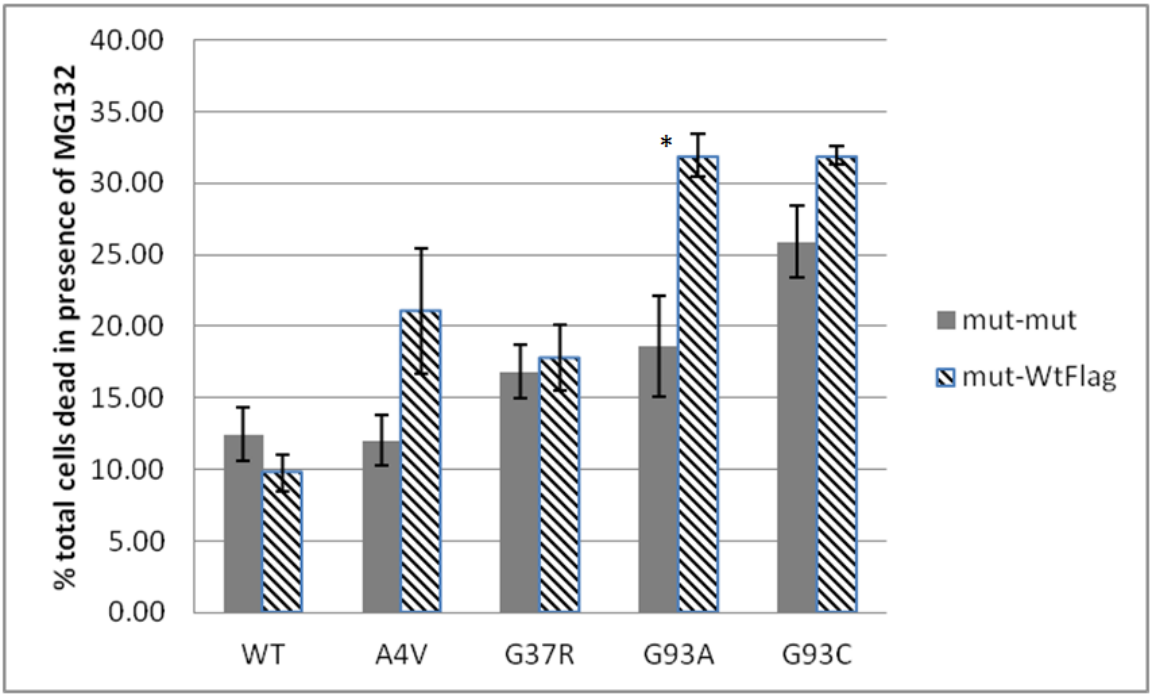


Figure 4.6: Presence of wild-type hSOD1 increases mutant hSOD1 vulnerability to proteasome inhibition. CHO cells were transiently transfected with equal total levels of mutant (mut-mut) or mutant and wild-type-3xFLAG (mut-WtFLAG) hSOD1 DNA and allowed to express for 24 hours before being exposed to 2.5 μM MG-132 for 24 hours. Viability was assessed via membrane integrity. Values represent the means of 3 independent experiments ± SEM. Statistical comparisons are mut-WtFLAG vs. the mut-mut condition (* = $p < 0.05$).

4.4: Discussion

We present data correlating the toxicity of mutant hSOD1 proteins, under a number of different conditions, with their relative solubilities. We demonstrate that under conditions of stress induced by proteasome inhibition, the most easily solubilized mutant proteins are also the most toxic, and that targeted re-folding of improperly folded, soluble mutant protein ameliorates this toxicity, even in the presence of less soluble SOD1 species. Furthermore, we demonstrate that increasing mutant hSOD1 solubility by co-expressing wild-type hSOD1 increases cell toxicity, as well as sensitivity to the effects of proteasome inhibition. The mutants demonstrating the greatest increase in solubility when co-expressed with wild-type hSOD1 also demonstrated the greatest increase in toxicity. This toxicity was rescued by inducing molecular heat shock chaperones, suggesting that the toxicity of mutant hSOD1 is related to levels of readily soluble misfolded hSOD1. It may be that the less soluble hSOD1 serves a protective function by sequestering the more soluble toxic species (figure 4.7). This less soluble fraction of SOD1 may include protein aggregates, though our experimental data do not address this issue. However, there is precedent for the hypothesis of protective sequestering of toxic proteins in other neurodegenerative diseases. Examples include models of Huntington's and Parkinson's disease, where pharmacologically enhancing aggregate size (and correspondingly decreasing levels of diffuse, soluble protein) increases the viability of mutant huntingtin- and synuclein-expressing cells (Finkbeiner et al., 2004; Bodner et al., 2006). Conformation-specific SOD1 antibodies are available; however, we chose not to use them because a) many are not thoroughly characterized, and b) separate mutants may not exhibit uniform misfolded conformations, decreasing the usefulness of such an approach.

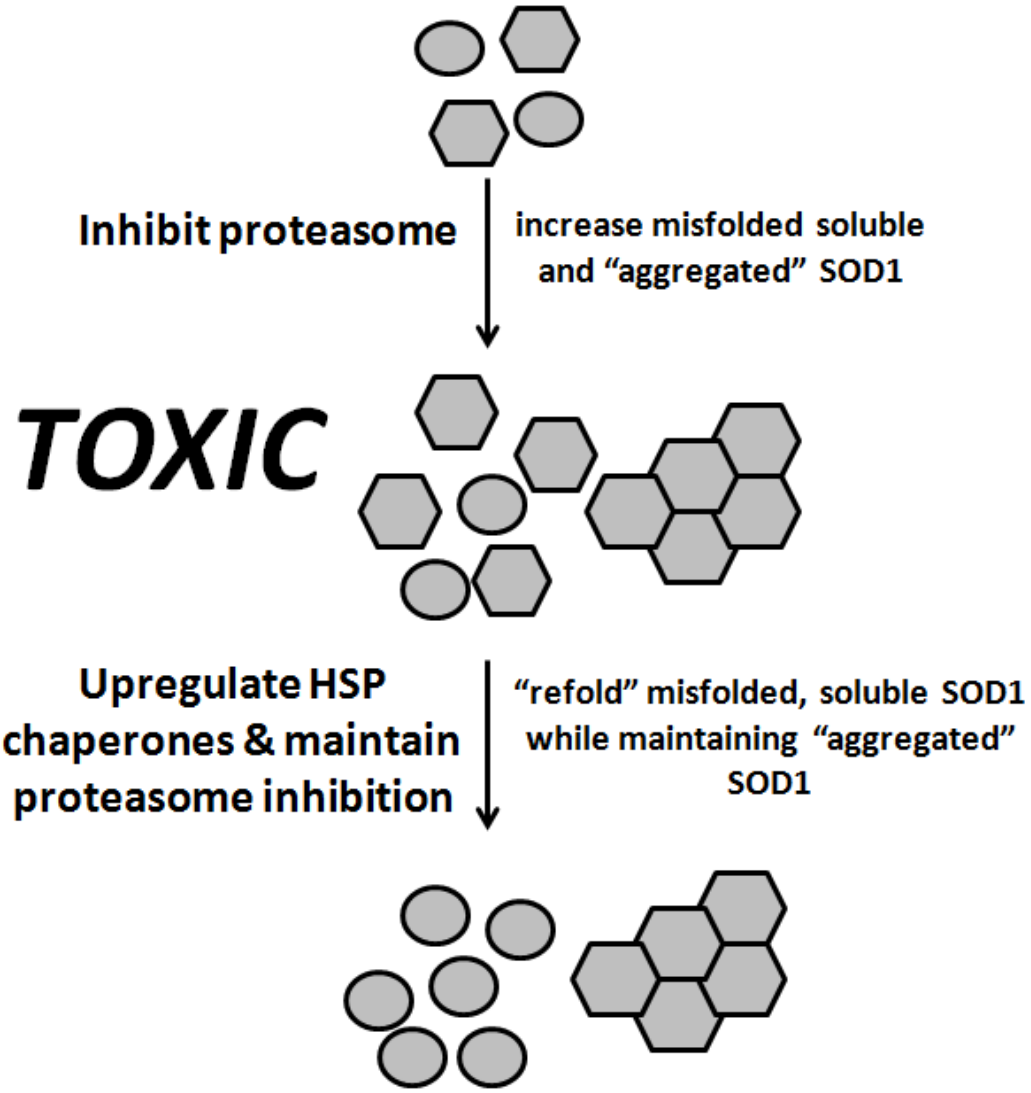

Inhibit proteasome
increase misfolded soluble and "aggregated" SOD1
TOXIC
Upregulate HSP chaperones & maintain proteasome inhibition
"refold" misfolded, soluble SOD1 while maintaining "aggregated" SOD1

Figure 4.7: Misfolded and soluble SOD1 is more toxic than aggregated SOD1. Mutant SOD1 is initially present as soluble, natively folded (circles) and soluble, misfolded (hexagons) protein. Proteasome inhibition slows protein turnover, increasing levels of soluble, misfolded and poorly soluble, potentially aggregated SOD1 (hexagon conglomerates) and is associated with increased cell toxicity. Up-regulating heat shock protein chaperones while maintaining proteasome inhibition targets soluble, misfolded protein for re-folding but does not decrease levels of poorly soluble, aggregated SOD1 and is associated with low levels of cell toxicity. The presence of poorly soluble, aggregated SOD1 alone is not enough to initiate toxicity; in contrast, misfolded, soluble SOD1 must be present for cell toxicity.

Our findings are consistent with previous data suggesting that the toxic hSOD1 species is localized in the soluble protein fraction (Zetterstrom et al., 2007; Wang et al., 2009a). There is debate over when protein aggregates first appear in rodent models of disease, but there is a general consensus that their presence drastically increases *after* the animals are already symptomatic (Johnston et al., 2000; Wang et al., 2002a). As detectable symptom onset is actually preceded by tissue pathology (*e.g.* denervation of the neuromuscular junction), factors other than protein aggregation are likely instrumental to disease initiation (Fischer et al., 2004). Indeed, both hydrophobic, soluble misfolded mutant hSOD1, and soluble mutant hSOD1 oligomers are present in pathologically affected tissue prior to symptom onset (Zetterstrom et al., 2007; Wang et al., 2009a). Furthermore, these protein species are selectively increased in pathologically affected tissue leading up to disease onset, supporting a role for soluble misfolded protein in disease initiation (Zetterstrom et al., 2007; Wang et al., 2009a).

A study on mutant SOD1 aggregation and toxicity utilizing live-cell imaging in PC-12 cells showed that those cells expressing mutant SOD1 aggregates were more likely to die than cells exhibiting a diffuse form of the protein (Matsumoto et al., 2005). However, toxicity was also observed in cells lacking aggregates, suggesting that aggregation is not necessary for cell death. A separate study using relative fluorescence of GFP-tagged SOD1 as a proxy for proper SOD1 folding showed that mutants with the most disrupted folding were also most sensitive to oxidative stress (Zhang and Zhu, 2006). There is also evidence that increasing the presence of molecular chaperones ameliorates mutant SOD1 toxicity both *in vitro* and *in vivo* (Bruening et al., 1999; Kieran et al., 2004; Batulan et al., 2006; Kalmar et al., 2008). However, these previous experiments could not differentiate the effects on

viability of aggregates versus soluble aggregate precursors. Our findings confirm the differential effects of separate SOD1 solubility states, providing further insight into the nature of the mutant SOD1 protein that is responsible for cytotoxicity.

The observed effect of wild-type hSOD1 on both mutant hSOD1 solubility and toxicity is consistent with that reported in other studies (Witan et al., 2008; Prudencio and Borchelt, 2009; Witan et al., 2009). Both solubility and toxicity of mutant hSOD1 was enhanced by the presence of wild-type protein. Ours is the first study showing that the increased solubility generated by co-expression of wild-type hSOD1 correlates with the relative increase in toxicity and that the enhanced toxicity can be attenuated by refolding the soluble misfolded protein by up-regulating heat shock chaperones. It may be that the wild-type protein somehow stabilizes soluble mutant protein, thereby increasing its toxic effect on the cell, and that inducing heat shock chaperones rescues this effect by stimulating re-folding of the stabilized, misfolded mutant protein. The mechanism through which wild-type hSOD1 interacts with the mutant protein is still unknown; separation of tagged SOD1 species through electrophoretic gels has suggested that co-expressed wild-type and mutant hSOD1 form a heterodimer (Witan et al., 2008; Witan et al., 2009), yet immunocytochemical studies showed that these species do not intimately interact (Prudencio et al., 2010). Even so, the presence of wild-type hSOD1 exacerbates mutant hSOD1-induced toxicity both *in vitro* and in rodent models, and has even been shown to initiate disease in otherwise unaffected A4V-expressing mice (Deng et al., 2006; Wang et al., 2009b; Prudencio et al., 2010).

Our findings showing enhanced solubility and toxicity of A4V in the presence of wild-type hSOD1 may provide insight into the A4V paradox. It is well-established that

humans heterozygous for the A4V mutation (no homozygous A4V humans have ever been reported) have one of the most rapidly progressing forms of ALS, whereas mice expressing A4V do not develop disease unless it is expressed in the presence of wild-type hSOD1 (Deng et al., 2006). Similarly in our studies, A4V-expressing cells were the least toxic in the context of proteasome inhibition, yet when co-expressed with wild-type hSOD1, A4V itself became toxic to the cell and the toxicity was further increased in the presence of a proteasome inhibitor (figures 4.5, 4.6). It thus appears that the presence of wild-type hSOD1 enhances mutant hSOD1 toxicity, possibly by stabilizing a soluble, misfolded form of the protein and/or inhibiting sequestration of toxic protein into aggregates. It is conceivable that the stabilization of mutant hSOD1 by wild-type hSOD1 accounts for the toxicity of A4V in heterozygous humans.

In conclusion, these data add to the growing literature implicating soluble, misfolded protein as the most cytotoxic protein species in various neurodegenerative diseases. We demonstrate that the toxicity and solubility of different SOD1 mutants are correlated *in vitro*, with the most soluble mutants also being the most toxic, and that this toxicity is attenuated by targeting improperly folded soluble protein for refolding, independent of the presence of less soluble SOD1 species. We further demonstrate that increasing mutant SOD1 solubility both induces toxicity and increases sensitivity to proteasome inhibition *in vitro*, and that this toxicity can be tempered through up-regulating molecular heat shock chaperones. These results support the hypothesis that toxic mutant hSOD1 is localized to the soluble fraction, and that a reduction in solubility may function as a protective, compensatory mechanism to sequester away this protein species. Future studies focusing on improperly folded soluble hSOD1 may shed light on the pathogenesis of ALS and other protein misfolding disorders.

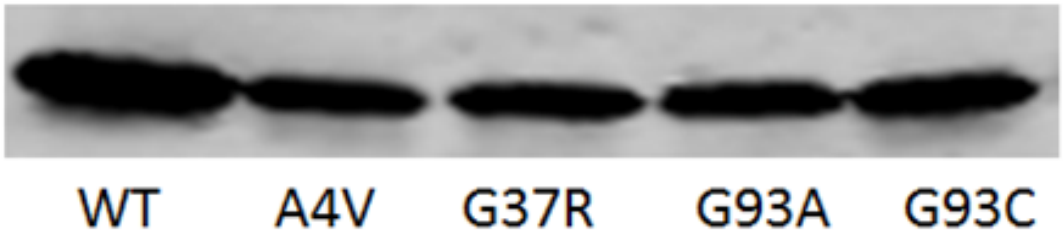


Figure 4.S1: hSOD1 mutants are expressed at equal levels. Transiently transfected CHO cells expressing mutant hSOD1 were separated through immunoblot and probed with a pan-SOD1 antibody. The mutant hSOD1 proteins showed similar levels of expression, the wild-type (WT) hSOD1 protein expressed at a higher level than all the mutants.

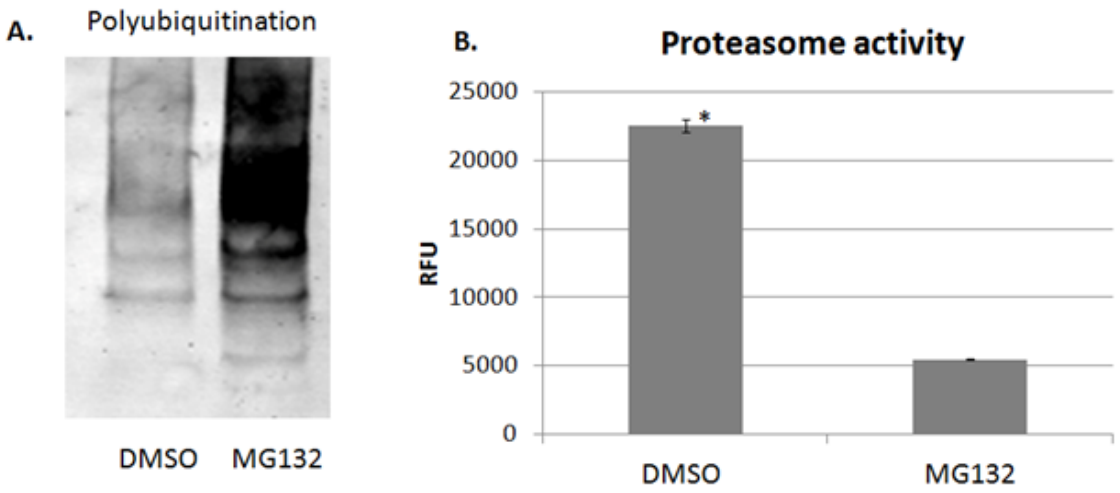


Figure 4.S2: MG-132 administration for 24 hours inhibits the proteasome. CHO cells were treated with 2.5 μM MG-132 or DMSO vehicle for 24 hours. A) Protein homogenates were separated through immunoblot and probed for polyubiquitination. MG-132 increased levels of polyubiquitin, consistent with an inhibited proteasome. B) 20S proteasome activity was measured through a fluorogenic, commercial assay. Proteasome activity was decreased in the presence of MG-132. Values represent the means ± SEM, and significance is relative to MG-132 treatment (* = $p<0.0001$).

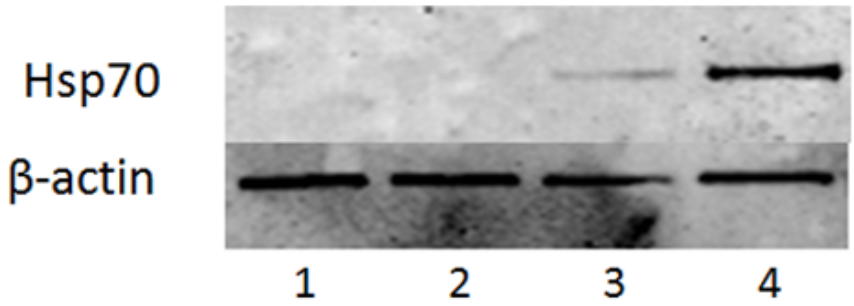


Figure 4.S3: Geldanamycin upregulates Hsp70 in stressed cells. CHO cells transiently expressing A4V were treated with (1) MG-132 for 24 hours (2) geldanamycin for 24 hours (3) MG-132 for 48 hours or (4) MG-132 for 48 hours with geldanamycin for the last 24 of those hours. Protein was separated through SDS-PAGE, transferred to PVDF membrane and probed for Hsp70. β-actin levels demonstrate equal protein loading. Hsp70 levels increased slightly due to exposure to MG-132 for 48 hours, the additional presence of geldanamycin further upregulated the expression of Hsp70.

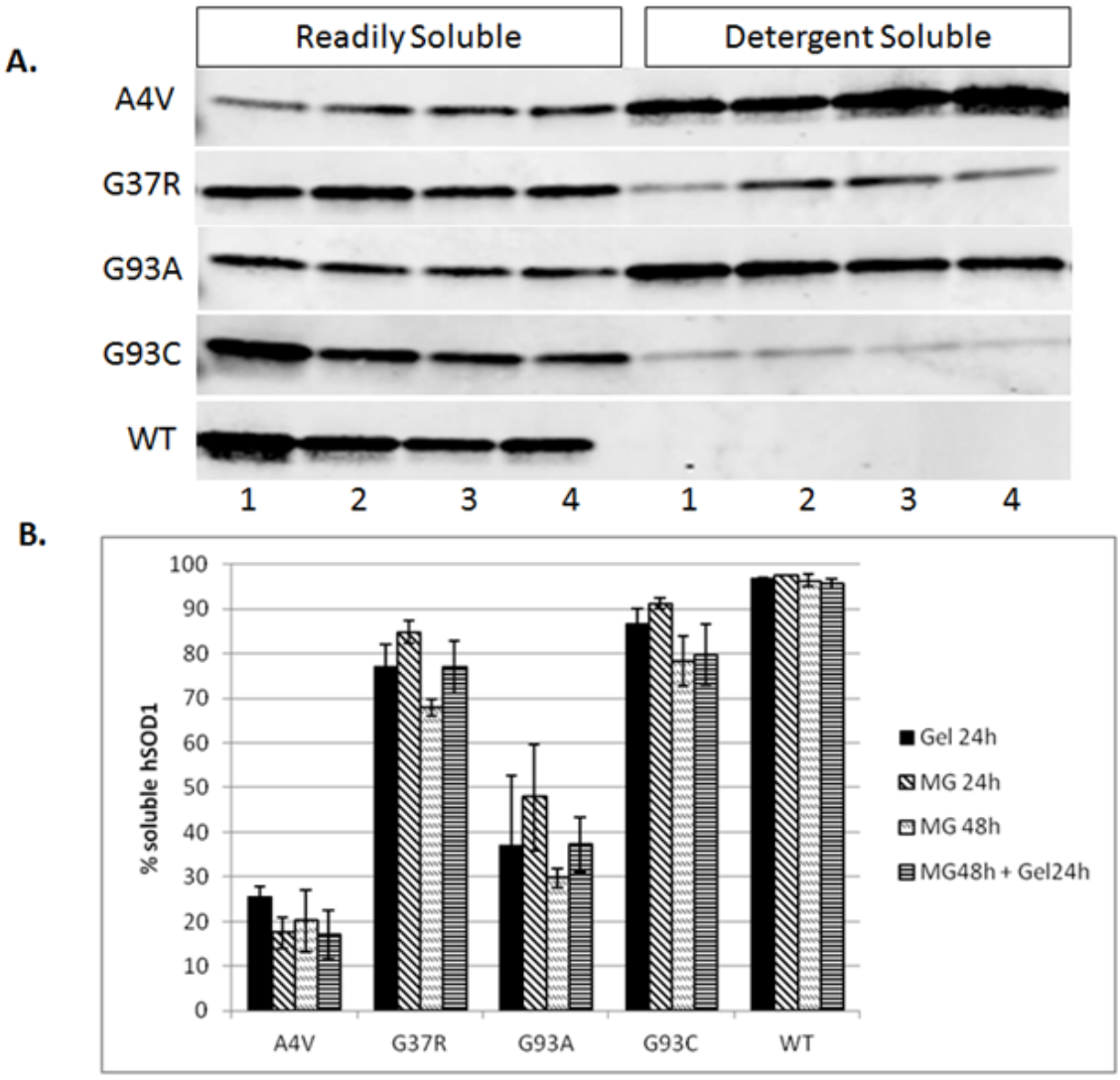

A.
Readily Soluble
Detergent Soluble
A4V
G37R
G93A
G93C
WT
1 2 3 4 1 2 3 4
B.
% soluble hSOD1
100
90
80
70
60
50
40
30
20
10
0
A4V
G37R
G93A
G93C
WT
Gel 24h
MG 24h
MG 48h
MG48h + Gel24h

Figure 4.S4: Relative solubility of each mutant is conserved when Hsp70 is upregulated in the presence of an inhibited proteasome. A) 24 hours following transfection, transiently expressing CHO cells were exposed to 2.5 μM MG-132 for 24 hours (1), 10 μM geldanamycin (Gel) for 24 hours (2), 2.5 μM MG132 for 48 hours (3), or 2.5 μM MG-132 for 48 hours with the cells simultaneously being exposed to 10 μM geldanamycin for the last 24 hours (4). Homogenates were separated into readily soluble and detergent soluble fractions and detected through immunoblot using a pan-SOD1 antibody. B) Levels of readily and detergent-soluble hSOD1 were quantified through optical densitometry and expressed as the percentage of readily soluble hSOD1 over the total hSOD1 for each condition. Relative solubility did not differ within each mutant regardless of drug administration. Values represent the means ± SEM.

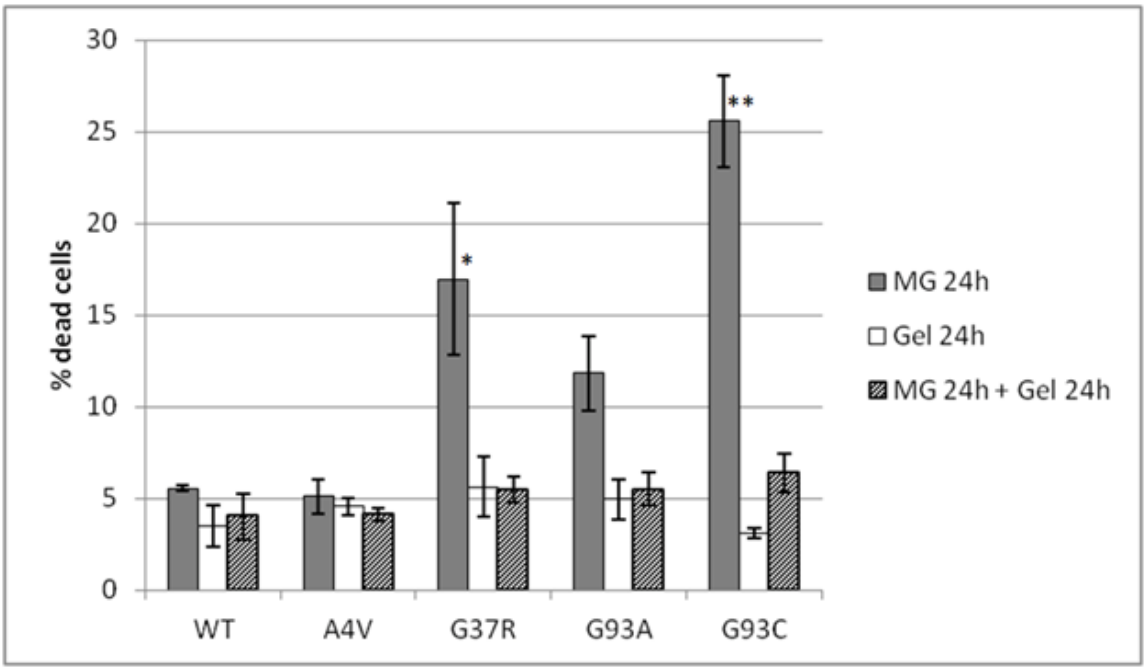


Figure 4.S5: Geldanamycin attenuates MG-132 induced toxicity. CHO cells transiently expressing wild-type (WT), A4V, G37R, G93A, or G93C hSOD1 were exposed to 2.5 μM MG-132, 10 μM geldanamycin (Gel), or both MG-132 and geldanamycin concurrently for 24 hours and toxicity was assessed via membrane integrity. Geldanamycin prevented the MG-132 induced toxicity. Values represent the means ± SEM, and significance is relative to MG-132 treatment for each mutant (* = $p<0.01$, ** = $p<0.001$).

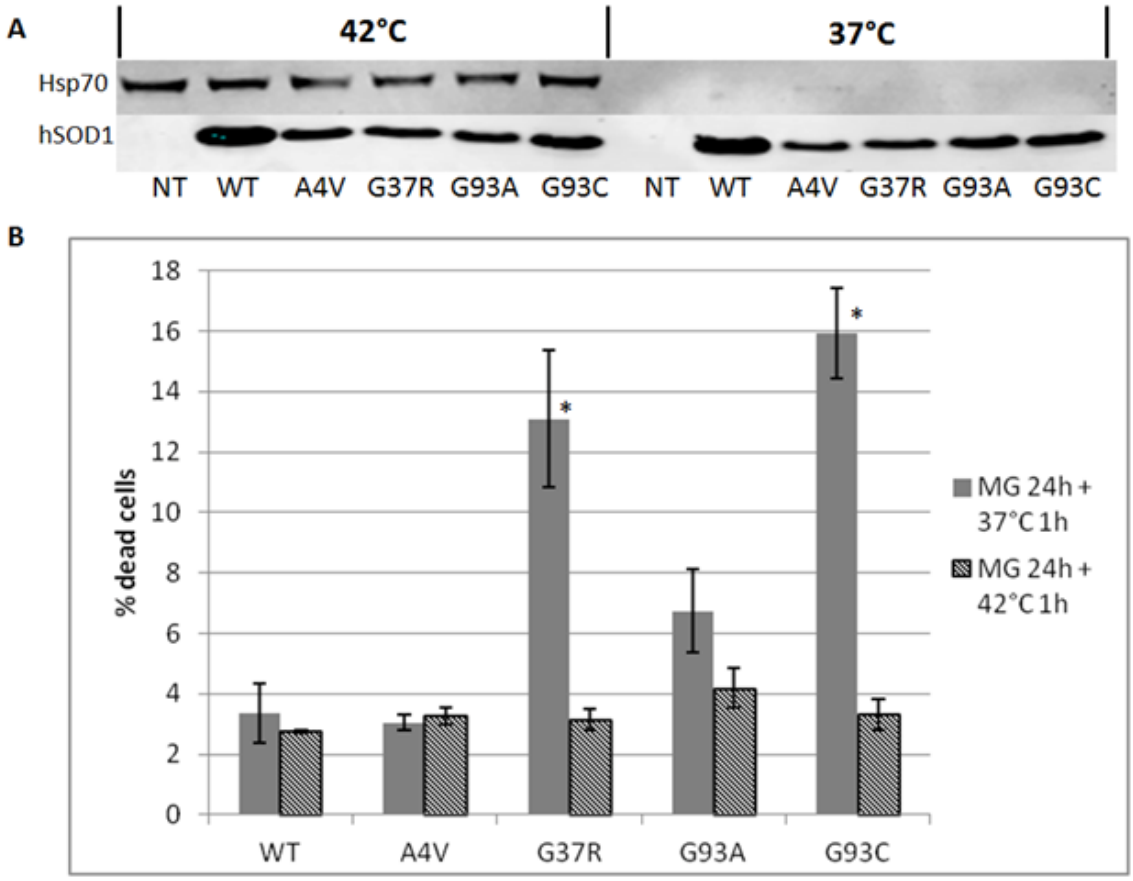


Figure 4.S6: Heat shock attenuates MG-132-induced toxicity. A) CHO cells transiently expressing wild-type (WT), A4V, G37R, G93A, or G93C hSOD1 were heat shocked at 42°C for 1 hour, or maintained at 37°C. The following day, cell homogenates were collected and probed for Hsp70 expression. B) CHO cells were exposed to 2.5 μM MG-132, heat shocked for 1 hour (or maintained at 37°C) and the following day assessed for viability via membrane integrity. Heat shock prevented the MG-132 induced toxicity. NT denotes non-transfected cells. Values represent the means ± SEM, and significance is relative to toxicity at 37°C for each mutant (* = $p<0.001$).

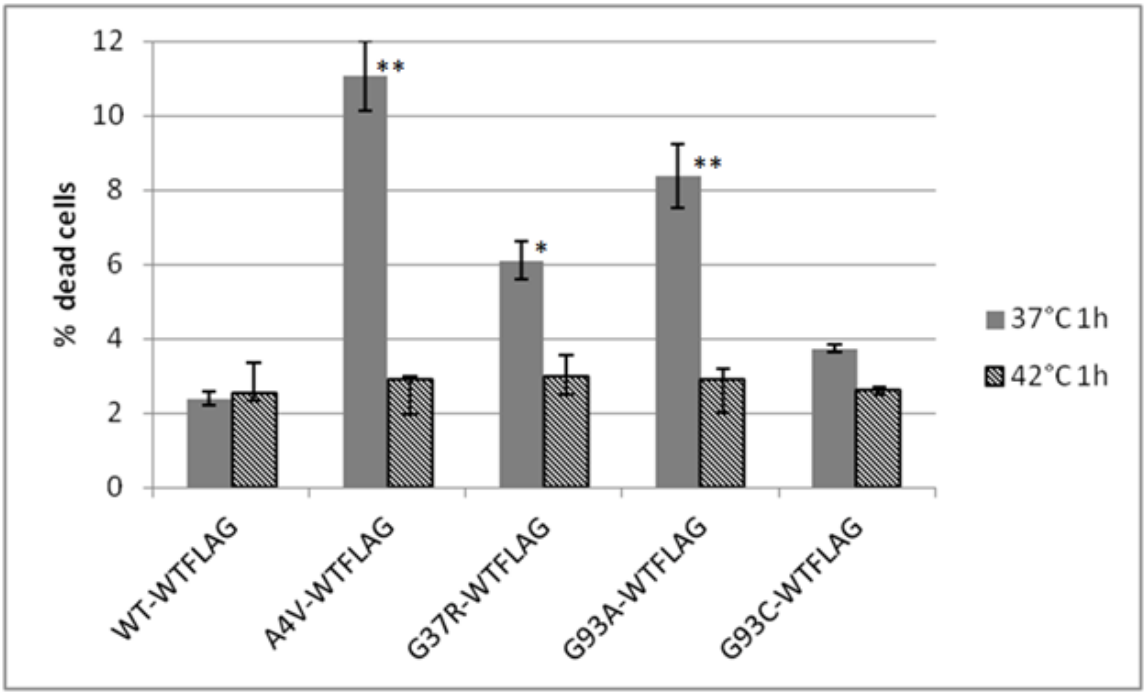


Figure 4.S7: Heat shock rescues wild-type-hSOD1 induced toxicity. CHO cells were transiently transfected with equal total levels of mutant and wild-type-3xFLAG (mut-WtFLAG) hSOD1 DNA, 24 hours later were exposed to heat shock for 1 hour, and the following day were evaluated for viability via membrane integrity. Heat shock at 42°C prevented WT hSOD1-induced toxicity. Values represent the means ± SEM, and significance is relative to toxicity at 37°C for each mutant (* = $p<0.05$, ** = $p<0.001$).

Chapter 5:

Discussion and Future Directions

This body of work is focused on the theme of cellular toxicity of mutant SOD1 proteins as it relates to the pathogenesis of ALS. Specifically, I have presented: 1) data from a family harboring a unique SOD1 mutation where the disease shows low penetrance; 2) experimental work in tissues from transgenic fALS mice and human autopsies showing the specificity of a unique antibody reagent that decorates mutant protein specifically in susceptible cell types; and 3) *in vitro* experimental data demonstrating that toxicity is associated with highly soluble mutant SOD1 protein, rather than less soluble protein. These data, though independent in their implications for toxicity, add important new information to the study of pathogenesis of ALS as it relates to SOD1 mutations and mutant SOD1 proteins.

Nearly two decades after the discovery that mutant SOD1 is linked to the development of ALS, the research and medical fields still do not possess a clear understanding of how the mutant protein causes disease. It was initially assumed that SOD1 linked ALS was due to a loss of function of the SOD1 enzyme and a treatment that restored or compensated for this lost function was expected to emerge rapidly; however, this has not been the case. Experimental evidence suggests that SOD1-fALS is not simply due to the loss of SOD1 activity. SOD1 transgenic mice develop a much more aggressive phenotype than mice lacking the enzyme, many SOD1 mutants retain full dismutase activity, and perhaps most compelling, SOD1 transgenic mice possess their own endogenous murine SOD1. Alternate explanations for mutant SOD1 toxicity have included an enzymatic toxic gain of function catalyzing the production of peroxynitrite (Ischiropoulos et al., 1992; Beckman et al., 1999; Estevez et al., 1999), deficits in axonal transport (Bosco et al., 2010),

impaired proteasome function (Bence et al., 2001; Kabashi et al., 2004; Bence et al., 2005; Kabashi and Durham, 2006; Kabashi et al., 2008), and mitochondrial dysfunction (Kawamata and Manfredi, 2008; Vande Velde et al., 2008). To date, none of these mechanisms have accounted for all aspects of SOD1 toxicity.

Protein aggregation is a unifying feature for a number of neurodegenerative disorders, including ALS, and therefore aggregates, the pathological hallmarks of disease, have historically been considered toxic. Disease associated proteins such as SOD1 have a propensity to aggregate in pathologically affected tissue, and due to this spatial co-localization aggregates are frequently regarded as harbingers of disease. However, recent methodological advances suggest this may be an oversimplified view of protein aggregation and that there are distinct roles played by classically defined aggregates, (*i.e.* insoluble conglomerates of protein), and their easily soluble, misfolded precursors. Specifically, the hypothesis that it is not aggregates themselves that are toxic, but instead their soluble, misfolded aggregate precursors, and furthermore, that aggregates form as a protective, compensatory cellular response to sequester toxic misfolded protein is increasingly being supported by the release of new data.

5.1: Identifying characteristics of toxic SOD1

The ultimate question under investigation in this project, the role of SOD1 solubility in ALS pathogenesis, is too large to be adequately addressed by one doctoral dissertation.

Encompassed within this question are more distinct points of contention: What are protein aggregates, and are all SOD1 aggregates the same? Are SOD1 aggregates toxic? What defines an oligomer? How do you define 'misfolded' proteins? Are misfolded proteins able to be refolded? How does protein quality control differ between cell types? Can toxicity be manipulated through manipulating solubility? What is the mechanism for cell death in mutant SOD1 expressing cells, and is this consistent with the mechanism of cell death in human SOD1-fALS? Is the mechanism for cell death in mutant SOD1 models and humans the same as in non-SOD1-fALS? What is the relevance of SOD1 studies to sALS? My dissertation has focused on two specific aspects of these questions - distinguishing toxic and non-toxic mutant SOD1 protein through immunodetection and relative solubility. These are almost certainly not the only ways to characterize toxic SOD1, and will invariably not encompass all toxic SOD1, however they may elucidate at least some characteristics of toxicity.

One of the great mysteries of ALS, and mutant SOD1 associated ALS in particular, is the specificity and distinct targeting of the motor system. Human fALS-SOD1 patients and transgenic rodent models of disease express the mutant protein at comparable levels throughout all tissues, yet the majority of cell populations are spared from disease. The implication is that if mutant/misfolded SOD1 is responsible for disease, then motor neurons must possess a decreased ability to cope with the mutant/misfolded SOD1. Interestingly, compared to other cell types, motor neurons exhibit a relatively high threshold for activating mechanisms important for protein quality control (Batulan et al., 2003). From a pathological perspective, the ability to distinguish toxic from innocuous mutant SOD1 would allow

tracking of disease activity and perhaps even reveal the nature of SOD1 toxicity. In chapter 3, I present the characterization of an antibody (C4F6) that discriminates between pathologically targeted and spared tissue both in SOD1 rodent models of disease and in SOD1-ALS autopsy tissue. Despite extensive attempts, I was unable to identify the epitope C4F6 recognizes on toxic protein. However, I did discover that the majority of C4F6-immunoreactive protein is found in highly soluble protein fractions, supporting the premise that cytotoxic SOD1 may be easily soluble. Additionally, indirect evidence points towards C4F6 recognizing a misfolded conformer of the mutant protein, however, until the C4F6 immunoreactive epitope is identified and/or the C4F6 immunoreactive protein is purified and crystallized, we will not possess definitive evidence that this is the case.

In order to assess the hypothesis that the relative solubility of mutant SOD1 protein correlates with cytotoxicity, I designed a series of experiments comparing the solubility of four separate SOD1 mutant proteins and their relative cytotoxicity. In conditions of cellular stress, specifically proteasome inhibition, the most soluble SOD1 mutant ($SOD1^{G93C}$) was the most toxic in a cell culture model and the least soluble SOD1 mutant ($SOD1^{A4V}$) was the least toxic. While interesting, this finding alone was not highly informative. One of the difficulties encountered by past researchers in evaluating the independent roles of misfolded, soluble aggregate precursors and the less soluble aggregates is that the formation of aggregates necessarily depends upon the presence of aggregate precursors, or protoaggregates. Therefore, it has been difficult to parse out whether toxicity is due to protoaggregates, or the aggregates themselves. To circumvent this issue, I devised a paradigm in which the effects of less soluble protein could be tested in the absence, or

diminished presence, of soluble misfolded protein. It is important to note that in this paradigm, proteins are classified by solubility and that soluble protein may not actually be part of a defined 'aggregate.' Through promoting the refolding of highly soluble, misfolded protein while simultaneously conserving levels of less soluble protein, it was possible to observe the singular effect of less soluble SOD1. I found that when the misfolded, soluble protein was refolded, the less soluble SOD1 was not toxic in this paradigm. Even more compelling, increasing the relative solubility of each SOD1 mutant increased its cytotoxicity. Indeed, by co-expressing human wild-type and mutant SOD1, I was able to transform the $SOD1^{A4V}$ mutant, a relatively non-toxic (and less soluble) protein into a cytotoxic (and highly soluble) protein species. This toxic effect could similarly be ameliorated by induction of the heat shock response to promote refolding of improperly folded proteins. Therefore, I demonstrated not only that the most soluble SOD1 mutants were associated with increased cytotoxicity, but that less soluble SOD1 was *not* associated with cytotoxicity, unless soluble misfolded protein was present. Additionally, I showed that by increasing mutant SOD1 solubility, mutant SOD1-associated cytotoxicity also increased. Taken together, this series of experiments suggests that highly soluble, misfolded SOD1 is cytotoxic, and that it may be more cytotoxic than less soluble SOD1.

My conclusions regarding the relationship between solubility and toxicity of mutant SOD1 are certainly not without limitation. The most notable limitation is that this was a correlative study – the solubility of mutant SOD1 directly correlates with cytotoxicity, but that does not definitively demonstrate that variations in solubility are responsible for variations in cytotoxicity. A separate property of the mutants being investigated may in

actuality have been responsible for changes in toxicity and the ideal study would demonstrate that two mutants identical in every way except solubility have differential effects. Furthermore, the mechanism whereby the cells die is not identified. Increased solubility of mutant SOD1 may correlate with another, as of yet unidentified, mechanism of cell death. A series of experiments identifying the molecular pathway leading to a loss of viability in these cells would greatly benefit our understanding of mutant SOD1 linked pathology. The implication from the outlined experiments is that misfolded, soluble SOD1 is responsible for cell death, but although there is a great deal of evidence supporting the assertion that the SOD1 being investigated is misfolded, due to technical limitations I did not directly demonstrate protein misfolding. As outlined in chapter 1, there are many 'misfolding' specific antibodies available, including the C4F6 antibody described in chapter 3, but they all possess very notable limitations. None of these antibodies encompasses all misfolded conformations, and many of the antibodies themselves rely on indirect evidence that they detect misfolded protein. Therefore, while an abundance of secondary evidence suggests that misfolded, soluble SOD1 is instrumental in pathology, I do not possess direct evidence that a) the protein in question is misfolded and b) that this protein is causally linked to cell death.

During the course of my dissertation work, I was fortunate to have access to clinical data and ALS patients. One family in particular challenged the notion that increased solubility of SOD1 correlates with toxicity. As outlined in chapter 2, this family possessed a cysteine-to-serine mutation at the sixth amino acid ($SOD1^{C6S}$), and demonstrated both low disease penetrance and slowly progressive symptoms (Brotherton et al., 2011). Interestingly,

$SOD1^{C6S}$ is not the only mutation identified at this amino acid in symptomatic individuals – $SOD1^{C6F}$ and $SOD1^{C6G}$ have been reported as well (Morita et al., 1996b; Kohno et al., 1999). However, while $SOD1^{C6F}$ and $SOD1^{C6G}$ are highly insoluble, $SOD1^{C6S}$ is not (Cozzolino et al., 2008; Karch and Borchelt, 2008; Kawamata and Manfredi, 2008). If a decreased propensity for aggregation is associated with increased toxicity, then $SOD1^{C6S}$ would be predicted to reflect a highly aggressive form of disease. The fact that $SOD1^{C6S}$ individuals presented with a milder phenotype underscores one of the main theses of this dissertation – it is not simply that soluble mutant SOD1 is toxic, rather it is specific conformers of soluble, *misfolded* mutant SOD1 that are toxic. We do not know the tertiary structure $SOD1^{C6S}$; however, because serine shares a high degree of structural similarity to cysteine and is its closest homolog among the amino acids, it is possible that the 6S structure may not differ greatly from wild-type. This emphasizes the need to identify structural alterations in mutant and otherwise modified SOD1, and moreover, to identify distinguishing characteristics among toxic varieties of SOD1.

5.2: Future Directions

While this body of work provides evidence that soluble, misfolded SOD1 is linked to pathogenesis, it does not provide an answer to the question of what makes SOD1 toxic. This is a very important, and very complicated question, and scores of investigators have struggled with it for years. What this dissertation does do however, is suggest experiments that may begin unraveling this very complicated issue. By using C4F6-immunoreactivity as a proxy for SOD1 toxicity, the effect of a variety of parameters on SOD1 toxicity can be seen.

In particular, stimulating increased or decreased solubility and observing the effect on C4F6-immunoreactivity may indicate whether toxicity can be mitigated by promoting aggregation.

The problem of manipulating protein solubility while minimizing experimental artifact is difficult, and it is perhaps for this reason that previous solubility studies have been largely correlative, not causational. Artifacts of using proteasome inhibition to manipulate SOD1 solubility include the general, systemic effects of decreasing protein turnover such as an accumulation of non-related misfolded proteins and/or a compensatory increase in other protein degradation pathways (e.g. autophagy), and more specifically, an increase in soluble, misfolded SOD1 prior to its aggregation. Aggregation is a response to the presence of misfolded, soluble protein, but it has not yet been discovered how to induce SOD1 aggregation without first increasing the presence of misfolded, soluble SOD1 (Munch and Bertolotti, 2010). The Parkinson's and Huntington's fields have approached this problem with unique, highly specific small molecules. The small molecule 5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitro-quinoline, colloquially referred to as B2, promotes aggregate formation while simultaneously decreasing the presence of misfolded, soluble protein, resulting in preserved cell viability (Bodner et al., 2006; Outeiro et al., 2007). B2 was recently found to be a histone deactylase inhibitor, though it is still unclear exactly how B2 targets the aggregation of misfolded, soluble protein (Outeiro et al., 2007). It would be interesting to see if B2 had a similar effect on mutant SOD1 solubility, and moreover, if it was able to rescue toxicity and/or decrease C4F6 immunoreactivity. Specific small-molecule inhibitors are preferential to more general modes of inducing aggregation (*e.g.* lipid vesicles, proteasome inhibition) because they can target unique protein conformations without

universally altering protein homeostasis; a small-molecule inhibitor that could promote the aggregation of misfolded SOD1 without increasing SOD1 misfolding may elucidate the cytotoxic or protective nature of protein aggregates. Conversely, it would be informative to observe how inhibiting aggregation affected toxicity. If misfolded protein is compelled to exist in a soluble state and cannot accumulate into aggregates, what would be the effect on cell viability?

Several small-molecules that stabilize select (e.g. A4V, G93A) SOD1 mutants and prevent their aggregation have been discovered (Ray et al., 2005). These molecules target a hydrophobic cavity that becomes exposed at the dimer interface of some mutants, preventing their subsequent dissociation into monomers. Because SOD1 monomerization precedes its aggregation, (Khare et al., 2004; Rakhit et al., 2004) then by preventing dimer dissociation, these molecules prevent SOD1 aggregation (Ray et al., 2005). Based on the hypothesis that aggregation is protective, I would predict that this would increase toxicity. However, it may be that the toxic form of SOD1 actually exists in the monomeric state, which would not be achieved in the presence of such stabilizing small molecules. If this were the case, these small molecules could actually decrease cytotoxicity due to decreased protein monomerization. Therefore, experiments that utilize stabilizing small molecules to investigate the toxicity of soluble SOD1 must be carefully designed and interpreted.

There are strong experimental data supporting the idea that ALS progresses through a non-cell autonomous mechanism and furthermore, that this is mediated by the secretion of soluble, misfolded SOD1. In chimeric mice possessing both mutant SOD1 expressing cells

and non-transgenic cells, motor neurons expressing the mutant protein showed delayed pathology when surrounded by non-transgenic cells, and conversely, non-transgenic motor neurons surrounded by mutant SOD1 expressing cells demonstrated ALS-like pathology (Clement et al., 2003). A series of Cre-LoxP experiments demonstrated unique phenotypic effects when mutant SOD1 was expressed specifically in different cell types. For example, the selective excision of mutant SOD1 from motor neurons and microglia was shown to, respectively, delay symptom onset or extend the symptomatic phase of disease (Boillee et al., 2006). The role of astrocytes in modulating mutant SOD1 toxicity to motor neurons has been examined through several methods. *In vivo*, selective excision of the $SOD1^{G37R}$ gene from astrocytes extended the symptomatic phase of disease without affecting onset in rodent models of disease (Yamanaka et al., 2008), the transplantation of non-transgenic glial restricted precursors into $SOD1^{G93A}$ rat spinal cord attenuated motor neuron loss and extended the symptomatic phase of disease (Lepore et al., 2008), and conversely, the transplantation of $SOD1^{G93A}$ glial restricted precursors into non-transgenic animals resulted in motor neuron death and motor dysfunction (Papadeas et al., 2011). *In vitro*, mutant SOD1 expressing astrocytes secreted a soluble factor selectively toxic to motor neurons, but not to interneurons or dorsal root ganglia (Nagai et al., 2007; Di Giorgio et al., 2008), and it was recently demonstrated that astrocytes derived from actual ALS patients, both with and without SOD1 mutations, were selectively toxic to motor neurons, and moreover, that this toxicity was decreased by knocking down SOD1 expression in astrocytes (Haidet-Phillips et al., 2011). Taken together, these studies implicate a non-cell-autonomous mechanism for cell death in ALS that is mediated by soluble, secreted SOD1. It would be informative to investigate how stimulating aggregation in mutant SOD1 expressing glia, but not non-transgenic motor neurons, would affect motor neuron pathology. I would predict that the

mutant SOD1 in the glia is only toxic to the motor neurons when in a soluble, more readily secreted state, and that by increasing aggregation, its non-cell-autonomous cytotoxic effects can consequently be ameliorated. A separate experiment utilizing small molecules to prevent aggregation in mutant SOD1 expressing glia may conversely exacerbate pathogenesis.

5.3: Therapeutic Implications

The development of therapeutics for ALS is hindered by a lack of understanding of disease mechanisms, and thus current therapies are largely palliative. The ability to discriminate toxic from benign forms of mutant SOD1 may elucidate the nature of ALS pathogenesis, at least in people harboring SOD1 mutations, perhaps allowing for the transformation of toxic SOD1 into an innocuous form.

If soluble, misfolded SOD1 is cytotoxic, and the evidence increasingly indicates that it is, the next question is if that toxicity can be attenuated. The most obvious solution would be increasing protein turnover to clear the cell of toxic protein, however as outlined in chapter 1, a clinical study that inadvertently increased proteasome activity had to be halted early due to loss of lean muscle mass (Miller et al., 1996). Therefore, increasing general protein turnover appears to be detrimental and consequently, a more selective targeting of toxic protein for degradation is likely needed. Until recently, we have not possessed the tools to distinguish toxic from non-toxic protein, but with the advent of antibodies such as C4F6, we have gained the ability to identify pools of toxic protein. Perhaps the C4F6

antibody could be modified in such a way that it (and whatever it recognized) would be targeted to the proteasome. As such, it would effectively act as a shuttle to recognize, and then transport only the toxic protein for degradation. Alternately, perhaps it could be modified in such as way as to target C4F6-immunoreactive proteins for refolding with the help of molecular chaperones, or even target C4F6-immunoreactive protein to already formed aggregates, thereby minimizing cytosolic interaction with the toxic protein. We do not currently possess the technological capabilities to make these therapeutics a reality, however, as we do possess a tool to distinguish toxic and innocuous mutant protein, it is worth considering methods that take advantage of this capability.

5.4: Conclusion

The mechanism whereby mutant SOD1 influences cell viability has evaded researchers for years, and this may be partially related to the current difficulty in distinguishing toxic and innocuous protein. This dissertation addresses how to differentiate toxic and non-toxic protein through immunodetection and suggests that solubility may play a role in disease pathogenesis. If the toxic SOD1 species can be isolated, then perhaps we can begin to understand how it exerts toxicity, why motor neurons in particular are targeted, and how to ameliorate cell death. Future investigations into identifying toxic SOD1 and the role of SOD1 solubility are warranted, and may lead to valuable therapeutic insights, not just for ALS but for neurodegenerative diseases in general.

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