

REVIEW

Protein misfolding in the late-onset neurodegenerative diseases: Common themes and the unique case of amyotrophic lateral sclerosis

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ABSTRACT

Enormous strides have been made in the last 100 years to extend human life expectancy and to combat the major infectious diseases. Today, the major challenges for medical science are age-related diseases, including cancer, heart disease, lung disease, renal disease, and late-onset neurodegenerative disease. Of these, only the neurodegenerative diseases represent a class of disease so poorly understood that no general strategies for prevention or treatment exist. These diseases, which include Alzheimer's disease, Parkinson's disease, Huntington's disease, the transmissible spongiform encephalopathies, and amyotrophic lateral sclerosis (ALS), are generally fatal and incurable. The first section of this review summarizes the diversity and common features of the late-onset neurodegenerative diseases, with a particular focus on protein misfolding and aggregation—a recurring theme in the molecular pathology. The second section focuses on the particular case of ALS, a late-onset neurodegenerative disease characterized by the death of central nervous system motor neurons, leading to paralysis and patient death. Of the 10% of ALS cases that show familial inheritance (familial ALS), the largest subset is caused by mutations in the *SOD1* gene, encoding the Cu, Zn superoxide dismutase (SOD1). The unusual kinetic stability of SOD1 has provided a unique opportunity for detailed structural characterization of conformational states potentially involved in SOD1-associated ALS. This review discusses past studies exploring the stability, folding, and misfolding behavior of SOD1, as well as the therapeutic possibilities of using detailed knowledge of misfolding pathways to target the molecular mechanisms underlying ALS and other neurodegenerative diseases.

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Key words: Alzheimer's disease; Parkinson's disease; Huntington's disease; prion disease; protein aggregation; amyloid; folding pathways; folding kinetics; ageing; senescence.

Abbreviations: AD, Alzheimer's disease; AL, immunoglobulin light chain amyloidosis; ALS, amyotrophic lateral sclerosis; CD, circular dichroism spectroscopy; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; DSC, differential scanning calorimetry; fALS, familial amyotrophic lateral sclerosis; FFI, fatal familial insomnia; FUS, RNA-binding protein fused in sarcoma; GSS, Gerstmann-Sträussler-Scheinker syndrome; HCDD, non-amyloid heavy chain deposition disease; HD, Huntington's disease; LCDD, non-amyloid light chain deposition disease; NMR, nuclear magnetic resonance spectroscopy; PD, Parkinson's disease; sALS, sporadic amyotrophic lateral sclerosis; sFI, sporadic fatal insomnia; SPR, surface plasmon resonance; TDP-43, TAR DNA-binding protein 43; TSE, transmissible spongiform encephalopathy; TTR, transthyretin; vCJD, variant Creutzfeldt-Jakob disease.

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HUMAN LIFESPAN AND THE LATE-ONSET NEURODEGENERATIVE DISEASES

In the past century, great strides have been made in combating many classes of human disease, resulting in major improvements in quality of life and life expectancy in both developing and developed nations.^{1,2} Major inroads have been made against the viral infections that have plagued humanity for centuries: vaccination has completely eliminated smallpox and one of three polio strains worldwide, and has decimated the incidence of polio, measles, mumps, and many other infectious diseases in most places.^{3–8} New vaccination efforts may soon confer the same fate on other childhood diseases, such as the chicken pox, that were universally suffered through as little as 20 years ago,^{9,10} and while influenza remains a major killer worldwide, the annual flu vaccine has also begun to reduce fatalities.^{11,12} Simultaneously, vaccines, antibiotics, and better medical care have revolutionized the prevention and treatment of bacterial infections that have long been major scourges, including cholera, leprosy, and tuberculosis.^{13–19} Although access to medicine is a persistent problem for a considerable subset of humanity,^{20–23} and while the emergence of new or therapy-resistant infectious diseases continues to be a threat,^{19,24} for the first time in human history, an eighty-year lifespan has ceased to be an upper limit achievable by a lucky few, and has become a realistic expectation for many in the developed world.^{1,2,25} Worldwide, the average life expectancy has now passed 68 years, reflecting major advances in human health in the developing world as well.²

Today, the major challenges for health care providers in industrialized nations are the diseases of ageing, and it is these that account for the vast majority of deaths. Cancer is now the top killer of Canadians, followed by heart disease, stroke, and lung disease.^{26,27} In the United States, the rank order is different, with heart disease leading cancer; however, both nations share the same four leading causes of death.^{28,29} In all four categories, major advances in diagnostic technology, in screening, in minimally invasive surgery, in tissue and organ transplantation, and in other therapies have helped to give patients suffering from these classes of disease a fighting chance.

Nevertheless, while the average life expectancy continues to rise, the maximum human lifespan still seems fixed at the value that it has held since ancient times: 80 to 90 years.^{2,25} Although modern medical advances, in combination with societal advances that have ensured better nutrition and safer living and working conditions, have removed many of the obstacles to reaching this limit, it seems that there exist inherent ageing processes that medicine still cannot address that cap achievable human lifespan (see Fig. 1). Several hypotheses for this

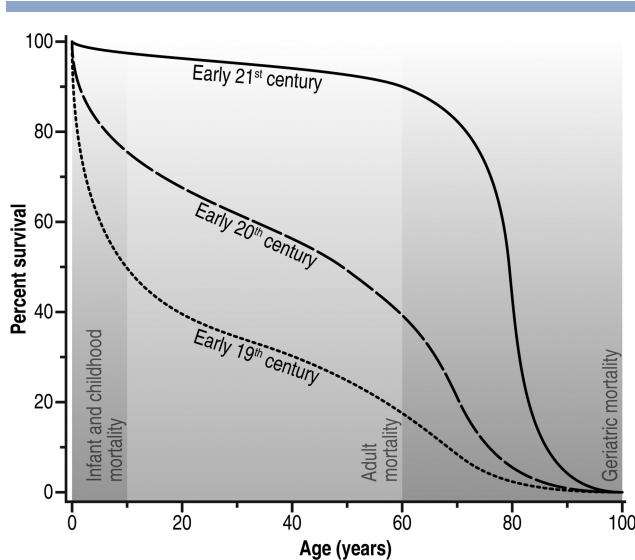


Figure 1

Survival curves for populations in developed nations at various points in human history. In the early 19th century and earlier (dotted curve), infectious disease and other factors resulted in very high infant and child mortality rates, as well as considerable adult mortality prior to age 60. Social and medical advances made in the 19th and 20th centuries reduced infant, childhood, and adult mortality considerably (dashed and solid curves), resulting in a much higher average life expectancy. Nevertheless, the maximum human lifespan remains largely unchanged. Graph drawn with reference to Figure 58-1 in Ref. 25 and to Ref. 2. Although the exact shape of these curves varies from nation to nation, this general trend—an increase in mean, but not maximum, life expectancy—has been a global phenomenon.

limit exist. It is commonly posited that it may be due to inherent limitations in the number of cycles of division that a somatic cell can undergo, possibly due to telomere shortening or other molecular mechanisms.³⁰ This, however, does not explain late-onset neurodegeneration, in which central nervous system (CNS) neurons, which have survived without dividing for decades, suddenly start to die. Arguably, it is the late-onset neurodegenerative diseases, which feature such neuronal death, that represent the greatest challenge for modern medicine. Moreover, it is possible that the molecular mechanisms underlying these diseases will prove central to the broader question of why we age.

The late-onset neurodegenerative diseases, which include Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), Creutzfeldt-Jakob disease (CJD), and the other transmissible spongiform encephalopathies (TSEs), and amyotrophic lateral sclerosis (ALS), are broadly characterized by onset late in life, progressive pathology involving the death of CNS neurons, and an invariably grim prognosis.^{25,31–35} Of these diseases, AD is now one of the top 10 causes of death of Canadians, and both AD and PD are among the top 15 causes of death of Americans.^{26–29,36} Moreover, as life expectancies rise and other causes of death are more

successfully treated, more people will be afflicted by these late-onset diseases within their lifespans, and these will become more common causes of death.²⁵

Current treatment strategies for these diseases are extremely limited. A common approach is to try to compensate for neuronal loss by increasing levels of corresponding neurotransmitters in the CNS (i.e. without directly trying to slow or halt neurodegeneration). To this end, L-DOPA is useful for raising dopamine levels in the brains of PD patients, temporarily compensating for loss of dopaminergic neurons.^{37,38} Similarly, acetylcholinesterase inhibitors raise acetylcholine levels in the cortex of AD patients, partially compensating for loss of cholinergic neurons.³⁹ These therapies offer temporary symptomatic relief, but do not affect the course of neurodegeneration or the ultimate outcome of the disease.^{37,39}

A handful of drugs have shown limited promise in slowing the neurodegeneration that characterizes the late-onset neurodegenerative diseases. These include riluzole and memantine, which can marginally slow the progression of ALS and AD, respectively, both by reducing the basal levels of glutamate excitotoxicity in the CNS.^{40,41} Riluzole adds a few months to the expected lifespan of an ALS patient, on average,^{42,43} while memantine has small effects on rates of cognitive decline in individuals with moderate to severe AD.^{44,45} Because these drugs work by reducing the ever-present background sources of neurotoxicity, and do not address the fundamental mechanisms underlying the neurodegenerative diseases, it is highly doubtful that a more potent or “better” version of any of these could ever serve as a treatment capable of halting disease progression completely. Currently, there exists no effective strategy for curing this class of disease. Development of cures targeting the fundamental mechanisms underlying the late-onset neurodegenerative diseases will require a detailed understanding of these mechanisms.

This review discusses our current understanding of the molecular mechanisms underlying these diseases, with a particular focus on protein misfolding and aggregation—common pathological features linking these diseases. We examine the unique case of ALS, in which the unusual stability of one of the causative proteins has permitted extremely detailed characterization of disease-associated protein misfolding and aggregation mechanisms. The recent studies that elucidated these mechanisms are summarized, and we discuss the implications for the development of therapies able to target the causative mechanisms underlying ALS and other neurodegenerative diseases.

DIVERSITY OF THE NEURODEGENERATIVE DISEASES

The neurodegenerative diseases are diverse, manifesting with distinct sets of neurological symptoms. Some, such

as AD, HD, and the later stages of PD, have broad effects on cognition, memory, mood, and mobility.^{25,32,46–48} The TSEs affect cognition and many other brain functions, including regulation of mood, speech, balance, movement, memory, and sleep^{49,50} (with sleep particularly affected in individuals with fatal familial insomnia, or FFI, and sporadic fatal insomnia, or sFI^{51–53}). In most cases of ALS, as well as in some PD cases, cognition is largely unaffected; however, impairment of mobility is always a feature of these two diseases, although the nature of the impairment is different in ALS and PD.^{35,38}

The regions of the CNS affected in the various neurodegenerative diseases reflect their diverse manifestations. AD causes massive neuronal loss throughout the cerebral cortex, as well as in deeper structures like the hippocampus, accounting for the disease’s diverse effects.^{25,46} HD also affects much of the brain, but disproportionately affects the basal ganglia, and causes some degeneration of the cerebellum, which is largely spared in AD. These features are consistent with the uncontrolled jerky movements, or “Huntington’s chorea” seen in the disease, and with the balance problems that are common symptoms in addition to the cognitive decline.^{32,54} The TSEs also cause neurodegeneration in many parts of the brain, producing a distinctive sponge-like appearance (“spongiform” change) when the grey matter of the cortex is examined by light microscopy.^{49,50} In the particular cases of FFI and sFI, sleep dysregulation is attributable to more pronounced degeneration of the thalamus.⁵² Although both PD and ALS involve movement problems as the most central symptoms, the former is mainly caused by destruction of dopaminergic neurons in deep brain structures like the *substantia nigra* that are involved in the processing and initiation of movement,^{38,47} while the latter involves the death of motor neurons in the motor cortex of the brain and in the spinal cord, which relay signals from the brain to the voluntary muscles.³⁵ PD patients therefore have difficulty planning, initiating, and pacing movements, and execute movements with a characteristic hesitancy, tremor, and rigidity, while ALS patients develop progressive weakness leading to full-body paralysis without the lower-level movement-planning problems seen in PD.^{35,38,47}

The causes of the various neurodegenerative diseases are also diverse. Most AD cases appear to be caused by neurotoxic processes present in everyone, so that AD apparently afflicts anyone who lives long enough: by age 65, roughly 7% of people show AD symptoms, and by age 80, the afflicted portion of the population reaches 40%.²⁵ PD also occurs sporadically in the large majority of cases, but shows some correlation with certain lifestyle-associated risk factors, including head trauma and exposure to certain toxins, though the strength of some of these correlations has been questioned.^{38,55–57}

Although AD and PD are predominantly sporadic diseases, roughly 5 to 10% of total cases of these diseases show a clear inheritance pattern.^{58–61} In this respect, ALS is similar, with about 10% of cases showing clear familial inheritance (familial ALS, or fALS), usually with a dominant inheritance pattern, while the remaining 90% of cases arise spontaneously without any apparent genetic mutation or unambiguous environmental cause (sporadic ALS, or sALS).^{35,62,63} HD is unique in that all cases show familial inheritance.⁵⁴ The TSEs represent the most complicated case: they can be inherited (as in the cases of FFI, hereditary CJD, and Gerstmann–Sträussler–Scheinker syndrome or GSS), can arise spontaneously (as is apparently the case in classical CJD and sFI), or, uniquely, can be acquired *via* exposure to an infectious agent (as in the case of the variant CJD, or vCJD, that resulted from consumption of beef from cows afflicted by bovine spongiform encephalopathy).^{49,50,64,65}

COMMON FEATURES OF THE NEURODEGENERATIVE DISEASES

Despite this diversity, these diseases possess a shared feature that is thought to represent a common molecular mechanism for pathology: the accumulation of insoluble proteinaceous material in intra- or extracellular aggregates within the CNS.^{31,33,64,66,67} In the spontaneous neurodegenerative diseases (e.g. classical CJD, most cases of AD, and sALS), it is thought that either low-probability stochastic processes can cause formation of a critical nucleus for aggregation of the specific causative protein over very long time-scales, or that ongoing basal rates of deposition of aggregates of the causative protein may result in accumulation of sufficient material for neurotoxicity over similarly long time-scales.^{50,65,68} Those diseases that show familial inheritance (e.g. HD, the familial TSEs including FFI and GSS, and 5 to 10% of PD, AD, ALS, and CJD cases) generally involve genetic mutations that promote aggregation of the causative polypeptide or protein.⁶⁹ For example, HD is caused by expansions of CAG repeat sequences in the *HTT* gene, resulting in extended glutamine repeat regions in the huntingtin protein encoded by this gene.^{54,69} These longer polyglutamine sequences are known to confer on this protein an increased propensity to aggregate.^{70,71} In some cases, the causative mutations are in proteins that alter the processing of the aggregation-prone polypeptide, as in the case of the *PSEN1* and *PSEN2* mutations that alter the average length of the amyloid- β (A β) polypeptide produced by the γ -secretase complex (a protein complex that includes presenilins 1 and 2, the products of the *PSEN1* and *PSEN2* genes).^{60,61,72} This results in enhanced A β aggregation and an early-onset AD phenotype.⁶⁰ In the unique case of the TSEs, the prion protein (PrP), which is the

causative agent in all TSEs, is known to be able to exist in an aggregation-competent state (termed the PrP^{Sc} state, for “scrapie,” an ovine TSE) distinct from its normal, biologically benign state (the PrP^C state, for “cellular” PrP)⁶⁸. PrP^{Sc} can promote the conversion of benign PrP^C and its recruitment into neurotoxic aberrant oligomers and aggregates.^{49,50,65} Consequently, PrP conversion to the disease-causing state can be caused by PrP mutations that promote the PrP^{Sc} state (genetic TSEs such as FFI), by low-probability stochastic conversion of WT PrP^C to PrP^{Sc} (sporadic TSEs such as classical CJD), or by exposure to another organism’s PrP that has already been converted to the PrP^{Sc} state (TSE infection, as in vCJD).^{49,50,53,64}

Although it is an open question as to whether it is the aggregates themselves or soluble aberrant oligomers on the aggregation pathway that represent the neurotoxic species in the various late-onset neurodegenerative diseases,^{67,73,74} conversion to aggregation-competent states and self-assembly into aberrant neurotoxic species represents a common theme in these diseases, and provides a shared molecular mechanism for diverse diseases that can arise sporadically, be inherited, or be acquired through infection. The protein aggregation pathways also represent a common point for therapeutic intervention, provided that the mechanisms of aggregation and associated neurotoxicity can be understood in detail.

PROTEIN FOLDING, UNFOLDING, AND MISFOLDING IN HEALTH AND DISEASE

The ability of PrP to exist in both benign and disease-associated states has generated considerable interest, and is illustrative of another general feature of the late-onset neurodegenerative diseases: the proteins implicated in these diseases are generally capable of undergoing a transition to a disease-associated state as part of the aggregation cascade that gives rise to neurotoxic effects.⁶⁸ This transition is an alteration in the conformation of the protein termed “misfolding.” Misfolding is thought to expose amino acid residues that are normally buried in the native state, and which can engage in aberrant protein-protein interactions on exposure.⁷⁵ Although the term “misfolded state” is often vaguely defined, for the purposes of this review, a “misfolded state” will be defined as any partially-structured conformational state of a protein or polypeptide that is distinct from both the well-structured native state and the fully unstructured, conformationally heterogeneous unfolded state. Note that some polypeptides have no well-structured native state that they adopt normally *in vivo*. The A β polypeptide, for example, has some α -helical propensity, but still populates a highly heterogeneous set of conformations in aqueous solution.^{76,77} Despite the absence of a structured native state, self-assembly of these polypeptides can result in partially-structured states distinct

from the fully unfolded state, which can be termed misfolded states. Additionally, the terms “folded,” “unfolded,” “misfolded,” etc. can be applied individually to separately folding domains in multidomain proteins.

Intrinsically-disordered polypeptides aside, the majority of human proteins are thought to have well-ordered native states, and proper folding is essential to most proteins’ functions.^{67,78} Failure to fold properly can either cause a loss of normal function (e.g. a disordered active site can impair normal activity, in the case of an enzyme) or can confer novel, potentially cytotoxic properties—particularly the ability to engage in aberrant protein-protein interactions if the misfolded state exposes considerable hydrophobic surface area that is buried in the native state.⁷⁸ In the case of proteins implicated in late-onset neurodegenerative disease, this gain of toxic function as a result of protein misfolding seems to be a common theme. Self-assembly of misfolded protein subunits leads to aberrant soluble oligomers and insoluble aggregates, with one or more species along this aggregation cascade responsible for neurotoxicity.⁷⁹ Many possible toxic effects of aberrant oligomers and aggregates have been proposed. These include congestion of the ubiquitin-proteasome system or other protein degradation machinery,^{80–82} disruption of the plasma membrane or organellar membranes,^{82–85} coaggregation with signaling or transcription factors causing loss of function of other cellular components,^{82,86,87} and interference with mitochondrial function.^{88–90} It is likely that misfolded species have the potential to exert multiple toxic effects, though in many cases it is thought that soluble oligomeric species have greater capacity to cause damage than relatively inert, insoluble aggregates.^{67,84}

Regardless the downstream effects of misfolded aberrant oligomers or aggregates, it is important to understand the mechanisms by which these toxic species are generated in the first place. The initial misfolded species that starts the aggregation cascade is sometimes an on-pathway folding intermediate, making the mapping of folding pathways a useful first step in understanding aggregation mechanisms.^{67,78,79}

CHARACTERISTICS OF PROTEINACEOUS AGGREGATES IN DISEASE

Examination by a number of techniques has revealed certain properties common to many, but not all, disease-associated proteinaceous aggregates. Under the transmission electron microscope, these aggregates tend to resemble long, unbranched fibrils approximately 5 to 10 nm in diameter.⁹¹ Circular dichroism (CD) spectroscopy has revealed that misfolded proteins in these fibrils tend to be rich in β -sheets.⁹² X-ray fiber diffraction confirms a β -rich structure, with β -strands running perpendicular

to the fiber axis (a characteristic “cross- β ” structure).⁹¹ In addition to these physical properties, aggregates tend to possess certain tinctorial properties: they generally bind the fluorescent Thioflavin dyes, as well as Congo red.^{91,93} Proteinaceous aggregates stained with Congo red usually show characteristic green birefringence under crossed linear polarizing filters.^{91,93}

All the above features are defining features of a particular type of proteinaceous aggregate, termed “amyloid” due to a superficial resemblance to starch under the light microscope.^{91,93,94} The A β plaques seen in AD and the huntingtin aggregates characteristic of HD are both examples of classic amyloid,^{92,93} as are protein depositions seen in certain non-neurodegenerative diseases, including deposits in kidneys and other tissues seen in cases of immunoglobulin light chain amyloidosis (AL).^{91,93,95} Additionally, harsh treatments (e.g. sonication) can coax a large number of proteins that are not amyloidogenic *in vivo* to form amyloid *in vitro*. This suggests that the propensity to self-associate to form amyloid may be a common property of polypeptides that competes with native folding.^{96,97} Although high-resolution studies of amyloid structure are hindered by the insolubility of amyloid fibrils and by the enormous molecular weights of the species involved, recent solid-state nuclear magnetic resonance (NMR) work has suggested that amyloid fibrils generally consist of stacks of parallel β -sheets stabilized by intermolecular backbone-backbone interactions, with additional stability imparted by hydrophobic and electrostatic side-chain interactions.^{98–101} Adjacent subunits making up a fibril have the potential to be offset relative to one another, raising the possibility that the high chain entropy associated with registry heterogeneity—the large number of microstates potentially contributing to the aggregated state—could actually play a role in stabilizing an amyloid fibril. Surprisingly, however, evidence has emerged for in-register assembly of amyloid fibrils in at least some of the fibrils studied to date, which would suggest that amyloid fibrils are curiously well-ordered structures.^{99–101}

Interestingly, not all protein depositions possess features of amyloid. The light chains of immunoglobulins form amyloid and non-amyloid aggregates in AL and in non-amyloid light chain deposition disease (LCDD), respectively.^{95,102} In the latter case, although immunohistochemistry reveals deposits of light chains, no Congo red staining is observed, and the aggregates have a granular appearance by electron microscopy distinct from amyloid fibrils.¹⁰² More rarely, immunoglobulin heavy chains can also form non-amyloid aggregates, leading to heavy chain deposition disease (HCDD).¹⁰³

Within the realm of neurodegenerative diseases, there have been claims of non-amyloid proteinaceous aggregates in addition to the amyloid plaques seen in AD,^{104–106} though this claim has not seen wide acceptance. Generally, there has been a tendency to assume that all neurodegeneration-associated protein aggregation

produces amyloid. In the case of ALS, this has led to *in vitro* attempts to probe the misfolding of some of the known causative proteins by using harsh conditions, such as high-frequency vibration in the presence of a hydrophobic Teflon surface, which can promote amyloid formation.^{107–109} These studies have yielded information about the *in vitro* mechanisms by which these proteins can produce amyloid. However, it has been shown that comparable conditions can induce amyloid formation in many proteins that do not form amyloid *in vivo*, including myoglobin, albumin, and hisactophilin.^{96,110} This raised the possibility that the *in vitro* protein misfolding models being used to study ALS were yielding information on an artificial phenomenon not associated with disease. As it turned out, subsequent histological studies examining the proteinaceous aggregates seen within ALS patient motor neurons revealed that these aggregates show no staining with Congo red, no green birefringence under crossed polarizing filters, and Thioflavin S staining indistinguishable from background,^{111,112} indicating non-amyloid character. Examination of ALS inclusions by electron microscopy revealed granular fibrous material and amorphous material distinct from amyloid fibrils.¹¹³ It is now known that different subsets of ALS cases have protein inclusions with different dominant protein constituents: sALS cases typically show aggregates containing the TAR DNA-binding protein 43 (TDP-43),^{114–117} while different familial cases of ALS can involve aggregation of optineurin,^{118–121} of the RNA-binding protein fused in sarcoma (FUS),^{122–124} or of the Cu, Zn superoxide dismutase (SOD1).^{125–127} Remarkably, all sALS and fALS aggregates from patient tissues examined to date have non-amyloid tinctorial and morphological properties, indicating that aggregation of many different proteins gives rise to inclusions with common non-amyloid character in ALS.^{111,112} Thus far, there has only been one report of any material in ALS patient tissues possessing amyloid-like properties: Robinson *et al.* recently reported that a subset of spinal TDP-43 inclusions in some sALS patients shows weak Thioflavin S staining not seen in TDP-43 inclusions in the brain, which was previously undetected due to background lipofuscin auto-fluorescence.¹²⁸ Because Thioflavin dyes have been shown to bind soluble misfolded protein oligomers *in vitro*, this is not a definitive indicator of amyloid, but one that requires confirmation by another method.^{91,129} However, even in the absence of such confirmation, the variability in the affinity of TDP-43 inclusions for Thioflavin S is an interesting indication of additional structural complexity in ALS aggregates.¹²⁸

Ultimately, the current evidence suggests that ALS is the best example of a non-amyloid late-onset neurodegenerative disease, setting it apart from other known late-onset neurodegenerative diseases—and this means that the elucidation of protein misfolding mechanisms in ALS

will rely on the development of good *in vitro* systems able to produce aggregates with the non-amyloid characteristics of those seen *in vivo*.

The presence of a fully non-amyloid neurodegenerative disease raises interesting questions: where should we place this disease relative to the amyloid diseases in the taxonomy of disease? Do proteinaceous aggregates with amyloid character, of the sort seen in AD, HD, PD, and the TSEs, and non-amyloid proteinaceous aggregates of the sort seen in ALS, represent entirely different phenomena caused by fundamentally different mechanisms? Could there exist commonalities in the misfolding and aggregation cascades that still somehow give rise to proteinaceous aggregates with very different biophysical and tinctorial properties? If all protein misfolding and aggregation pathways underlying the major neurodegenerative diseases share common mechanisms, it becomes plausible to envision broad-spectrum therapeutics targeting these mechanisms that could one day be used to treat this class of disease. Alternatively, if fully distinct phenomena underlie the amyloid and non-amyloid neurodegenerative diseases, a “divide and conquer” approach may be called for. The answer to the question of the relationship between amyloid and non-amyloid protein misfolding and aggregation mechanisms could therefore have major implications for the development of strategies for treating the neurodegenerative diseases. ALS currently represents the only major neurodegenerative disease known to show exclusively non-amyloid deposition of misfolded protein. In order to understand the relationship between amyloid and non-amyloid disease, the details of the protein misfolding and aggregation mechanisms in ALS must be elucidated.

THE UNIQUE CASE OF AMYOTROPHIC LATERAL SCLEROSIS

Clinical presentation and pathology of ALS

Although the first medical descriptions of ALS cases likely date back to the 1820s,¹³⁰ Jean-Martin Charcot's 1874 report of the disease¹³¹ is widely considered the first detailed characterization, and is lauded for its completeness.^{130,132} Charcot carefully catalogued the large-scale physiological changes in ALS patients, including weakness progressing to total paralysis of the voluntary muscles, spasticity or rigidity, limb spasms, mechanical difficulties with pronunciation and swallowing, and eventual respiratory difficulty leading to death.^{130,131} He also documented the neurological functions that are preserved in the disease, including sensation, cognition, and, for the most part, control of the eyes, bowels, and bladder.¹³⁰ This characterization has largely survived unscathed into the 21st century, though it has recently been reported that subtle cognitive changes may occur in a large subset of ALS cases, mostly manifesting as

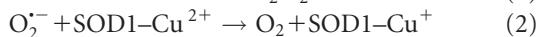
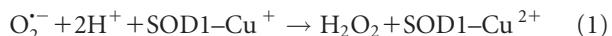
difficult-to-detect frontotemporal dementia symptoms (though this is still an area of controversy within the field).^{133–135} Epidemiological studies have also added new information about affected demographics, onset, and disease progression: we now know that males are disproportionately affected by sALS by a factor of about 3 to 2,⁶² that disease onset is on average around age sixty (though onset in the fifth through ninth decades of life is common),¹³⁶ and that half of ALS patients will die within three years of symptom onset.³⁵ The prevalence of the disease—about four to six cases per 100,000 population—is deceptively low, since ALS kills so rapidly.^{35,62} A better indication of its frequency is the lifetime risk of developing ALS, which is between 1:300 and 1:400.^{136–138} As mentioned previously, about 90% of cases (sALS) are of unknown cause; the remaining 10%, while clinically indistinguishable from sALS, show familial inheritance (fALS) suggesting a genetic cause, typically with a dominant inheritance pattern.⁶²

Charcot's characterization of tissue-level changes in the brain and spinal cord was also remarkably complete. From autopsy samples, he identified deterioration of the lateral columns of the spinal cord as a defining feature of the disease, which would later be determined to contain the axons of upper motor neurons that have cell bodies in the motor cortex of the brain.^{130,139} He also identified lesions affecting the anterior horn and anterior root, regions of the spinal cord later determined to contain lower motor neuron cell bodies and axons, respectively.^{130,140} Today, these are recognized as the defining cellular features of ALS: progressive destruction of both upper and lower motor neurons, with muscular atrophy a downstream effect of denervation.³⁵ Although Charcot had few patients on whom his characterization was based, we now know that ALS typically begins at a focus within the CNS and spreads, resulting in spread of symptoms in a manner that parallels the outward destruction of CNS tissue from the initial site of pathology.¹⁴¹ For example, a plausible disease course could involve weakness starting in the right leg that gradually spreads to the left leg and right arm, corresponding to loss of motor neurons nearest the site of initial pathology in the spinal cord. Progressive paralysis of the left arm, tongue, and face would be expected to occur later in the disease in this case since spinal motor neurons innervating these areas lie farthest from the site of initial pathology. Some have speculated that this spread from an initial focus may reflect propagation of protein misfolding by an as-yet undetermined mechanism, possibly resembling PrP^{Sc}-initiated misfolding of PrP^C.^{142–144}

Familial ALS and the Cu, Zn superoxide dismutase

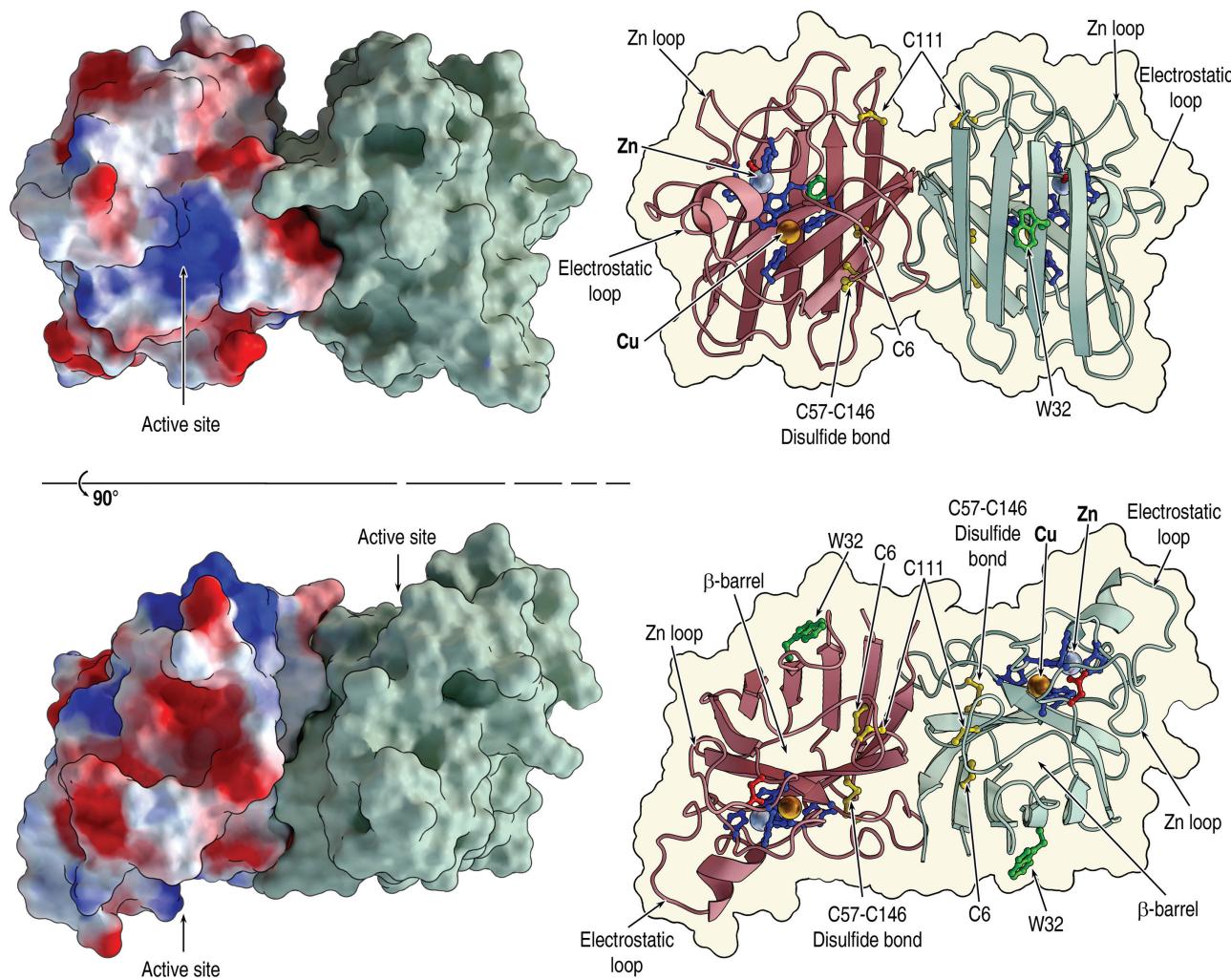
Although the majority of ALS cases show no genetic linkage, the inherited subset has provided the most

information about the molecular mechanisms underlying the disease. In 1993, it was discovered that about 20% of cases of fALS (the plurality) could be attributed to mutations in the *SOD1* gene, which encodes the Cu, Zn superoxide dismutase (SOD1).¹²⁵ This enzyme is one of the primary components of cells' defense mechanism against oxidative damage. It converts superoxide ($O_2^{\cdot-}$), a dangerously reactive free radical species produced in the mitochondrion as a byproduct of oxidative metabolism, into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2).^{63,145} The reaction involves a “ping-pong” mechanism in which the enzyme's active-site Cu atom cycles between the Cu^+ and Cu^{2+} states [Eqs. (1) and (2)]:^{63,145}



One hundred sixty-five unique ALS-causing mutations have now been documented,¹⁴⁶ the large majority of which are single amino acid substitution mutations causing a dominantly-inherited disease phenotype. These are scattered throughout the amino acid sequence of the enzyme. Interestingly, most ALS-causing mutations have little to no effect on SOD1 enzymatic activity.¹⁴⁷ Removal of SOD1 enzymatic activity in mice through knockout of the mouse *SOD1* gene results in a subtle neuromuscular phenotype, but not in the progressive and ultimately fatal neurodegeneration seen in ALS.¹⁴⁸ Co-expression of human SOD1 bearing an ALS-causing mutation alongside the endogenous WT mouse SOD1 does cause mice to develop a progressive ALS phenotype, however, and the G93A, G37R, and G85R mouse models have become standard models of disease.^{149–151} All of this suggests that the common property of ALS-causing SOD1 mutations is a gain of toxic function.

In the 1990s, early hypotheses about the nature of the novel toxic function focused on aberrant SOD1 activity. Various groups suggested that mutant SOD1 might gain the ability to allow new substrates to bind the active site, resulting in production of dangerous free radical species such as peroxy nitrite.^{152,153} However, in 2003 it was shown that murine expression of human SOD1 in which all Cu-binding amino acid residues had been mutated resulted in a rapidly-progressing ALS phenotype, even in the absence of known ALS-causing mutations,¹⁵⁴ suggesting that the gain of toxic function cannot be related to Cu-dependent enzymatic activity. It is now generally accepted that the common effect of ALS-causing SOD1 mutations must involve an increase in the propensity for SOD1 to misfold and to aggregate, though until recently, the molecular details of these processes remained obscure.⁶³

**Figure 2**

Structure of the Cu, Zn superoxide dismutase. Lower structures are rotated 90° about the horizontal axis parallel to the page relative to the upper structures. On the left, the SOD1 homodimer dimer is shown in surface representation, with one subunit in dull green and the other with colours representing charge distribution. The electrostatic funnel surrounding the SOD1 active site, which directs the negatively-charged O₂[−] to the catalytic Cu, shows considerable positive charge. On the right, the enzyme's β-barrel core structure is shown as a cartoon representation, with subunits coloured in dull red and dull green. Active-site Cu and Zn atoms are shown as spheres, and metal-binding histidine and aspartate residues are shown as blue and red sticks, respectively. W32 is shown in green, while cysteine residues are shown in gold. Coloured electrostatic surface, cartoon, and stick representations of chains A and B from PDB structure 1PU0 were exported to VRML format with PyMOL 0.99rc6, and rendered with Maya 2012 software and the mental ray renderer.

SOD1 structure and stability

SOD1 is a highly soluble, small, globular protein. As shown in Figure 2, the protein is a homodimer of two subunits, each of 153 amino acid residues, and each with an eight-stranded β-barrel core structure of the immunoglobulin-like fold type.^{155–158} (Interestingly, the proteins currently implicated in non-amyloid aggregation diseases LCDD and HCDD share this fold,^{102,103} though it is possible that this is coincidence, since immunoglobulin-like folds are extremely common.) Loop regions extend from the ends of the β-strands, two of which—the “Zn loop,” located between strands 4 and 5,

and the “electrostatic loop,” located between strands 7 and 8—form the active site.^{63,159} The active-site Cu is coordinated by four histidine residues (H46, H48, H63, and H120), three of which are located on β-strands 4 and 7, while the adjacent Zn is coordinated by three histidine residues (H63, H71, and H80) and one aspartate residue (D83), all of which are located in the Zn loop.¹⁵⁶ H63, located in the Zn loop, is a bridging histidine residue that coordinates both metals. As the active-site Cu cycles from the Cu²⁺ state to the Cu⁺ state, the coordination with H63 is lost. Although the binding sites for these metals are far from the dimer interface, the Zn

loop winds back across the surface of the β -barrel to form part of the dimer interface, allowing metal binding and oligomeric status to affect one another.^{160–162} Although the protein is predominantly a mix of β -sheet and random coil, binding of Zn^{2+} stabilizes a short α -helix in the electrostatic loop through electrostatic interactions with the helix macropole,⁶³ structuring both the electrostatic and Zn loops.¹⁶⁰

SOD1 possesses a single tryptophan residue, W32, which is exposed to solvent on the face of the β -barrel opposite to the active site. This residue's intrinsic fluorescence is quenched more when the protein is in its native state than it is when SOD1 is in its fully unfolded state. This is due to the highly polar environment created by N19 and E21 on the adjacent β -strand. As a result, a slight increase in fluorescence intensity is detectable when the protein transitions to the fully unfolded state and these interactions are lost.¹⁶³ There are also four cysteine residues in each SOD1 subunit, two of which (C57 and C146) are involved in an intramolecular disulfide bond that links the Zn loop and β -strand 8.¹⁵⁶ Of the remaining two, C6 is buried, while C111 is solvent-exposed near the dimer interface, with the γ sulfur 8.25 Å from its counterpart on the other subunit.¹⁵⁵ In SOD1 isolated from human sources, this is a possible site of post-translational modification. Glutathionylation has been observed here,^{164,165} as has an unusual five-sulfur polysulfane bridge linking the two subunits.^{162,166–169} The biological relevance of these post-translational modifications, if any, is currently unknown.¹⁷⁰ Interestingly, a 1969 work comparing the properties of human SOD1 purified from brain and liver detected the characteristic 325 nm absorbance peak which would be recognized later as a signature of the polysulfane bridge.^{162,166,167,171} This peak was only observed in the SOD1 from human brain, however, and not in liver SOD1.¹⁷¹ Other works from the same era also detected this absorbance peak in SOD1 from human blood.¹⁷² In addition to these C111 modifications, one group has reported possible phosphorylation at T2 and either T58 or S59, but this has not been reported elsewhere.¹⁶⁵

Both the thermodynamic and kinetic stabilities of SOD1 are truly exceptional. Differential scanning calorimetry (DSC) studies of disulfide-intact WT human SOD1 with Cu and Zn bound have variously reported melting temperatures of 88 °C,¹⁷³ 93 °C,¹⁷⁴ and 101 °C,¹⁷⁵ with differences likely attributable to variations in experimental pH and solution conditions, as well as possible heterogeneity in protein metal content. In comparison, most proteins unfold at temperatures only slightly above the physiological—typically in the 40 to 60 °C range.¹⁷⁶ (Note that some of these SOD1 DSC studies used a C6A/C111S pseudo-WT SOD1 variant which is not known to be ALS-associated. Mutation of these free cysteine residues prevents aberrant disulfide cross-linking during thermal denaturation, resulting in

better reversibility and less ambiguous DSC traces, while having nearly no effect on protein structure or thermodynamic stability.^{155,173}) Denaturant-induced unfolding studies monitored using CD spectroscopy or tryptophan fluorescence have revealed that SOD1 retains its native structure in up to about 3.2 M guanidine HCl, and takes days to equilibrate near the transition midpoint, indicating that the protein possesses incredible kinetic stability in addition to its remarkable thermodynamic stability.^{163,177}

The bound metals and the intramolecular disulfide bond all contribute greatly to SOD1's thermodynamic stability. Removal of metals to produce apo-SOD1 reduces the melting temperature to between 50 °C¹⁷⁸ and 59 °C,¹⁷⁹ depending on experimental conditions. Disulfide reduction on top of metal loss further reduces the melting temperature to 43 °C, resulting in marginal stability at physiological temperature, and causes the guanidine HCl unfolding midpoint to shift down to 1.5 M guanidine HCl from the value of 3.2 M reported for the disulfide-intact holo-protein.¹⁸⁰ Time-resolved acid denaturation studies have also indicated that both Cu and Zn contribute greatly to SOD1's kinetic stability, with Cu having the greater stabilizing effect under acidic conditions.^{181,182} In endpoint guanidine HCl-induced denaturation experiments, however, Zn binding had the greater effect on the thermodynamic stability of SOD1.¹⁸³ This apparent discrepancy may be due to the more facile protonation of Zn-coordinating histidine residues at low pH, resulting in reduced affinity for (and, consequently, stabilization by) Zn under acidic conditions.^{184,185} A synergistic relationship between Zn binding and SOD1 dimerization has also been established.^{160,162,183,186–188}

Following the 1993 discovery of *SOD1* gene mutations as a major cause of familial ALS,¹²⁵ many hypotheses were put forward as to the mechanism by which ALS-causing SOD1 mutations promote misfolding and aggregation. The simplest suggestion was that ALS-causing mutations have the common effect of reducing the global stability of natively-folded SOD1 (perhaps resulting in a perturbed or strained native state), thereby promoting misfolding.^{155,174,189–192} Reductions in affinity for bound metals, in propensity to form a stable intramolecular disulfide bond, or in the stability of partially metal-loaded, apo-, or disulfide-reduced states have also been proposed as possible “common denominators” of ALS-causing mutations.^{152,160,178,180,193–197} Surprisingly, none of these hypotheses proved to be the common feature linking all ALS-causing SOD1 mutations. Under physiological conditions, most ALS-associated mutant forms of SOD1 fold nearly as readily as the WT protein. Several crystallographic studies have revealed that most of the ALS-causing point mutations cause no major alterations to the native structure of SOD1, and that the few that do typically do so by disrupting metal binding,

resulting in disordered Zn and electrostatic loops (much like WT apo-SOD1) but an unperturbed β -barrel core.^{198–201} While many of the known ALS-causing SOD1 mutations do result in slight reductions in overall thermodynamic stability, corresponding to decreases in the T_m of holo-, apo-, or disulfide-reduced SOD1 on the order of 5 to 10°C,^{178,202} this results in a protein that is still far more stable than most proteins; moreover, this is not a common feature of all ALS-causing mutations. Indeed, some ALS-causing mutations, such as the H46R mutation, actually stabilize the apo- and disulfide-reduced states as compared with the WT protein, while others cause very little change in stability.^{193,203,204} It is now generally accepted that global destabilization of holo-, apo-, or disulfide-reduced SOD1 by ALS-causing mutations cannot explain the ALS phenotype.^{203,204}

Oxidative damage as a trigger for mutation-primed SOD1 misfolding

All of this raises a perplexing problem. On the one hand, both WT and mutant SOD1 are generally exceptionally stable, fold normally, and do not readily populate fully unfolded states; moreover, ALS mutations do not appear to have a common detrimental effect on global protein stability, on stability of apo- or disulfide-reduced states, or on protein structure. On the other hand, mutation somehow has the common effect of promoting neurotoxic protein aggregation, presumably through some sort of misfolding mechanism. Even more confusing is the fact that SOD1 is expressed ubiquitously, and at high concentration,²⁰⁵ yet only misfolds and aggregates in brain and spinal cord motor neurons, the CNS cells that show selective mortality in ALS.²⁰⁶ Additionally, the late onset of a disease caused by an abundant protein expressed from birth is difficult to explain. It has been proposed that the appearance of aggregates of misfolded mutant SOD1 late in life in ALS patients is attributable to age-related decline in motor neurons' ability to clear misfolded protein.^{207–209} Although this likely plays a significant role in the disease, even in the complete absence of the cellular protein degradation machinery *in vitro*, mutant SOD1 can be maintained at physiological concentration for long periods, requiring considerably elevated concentrations to show measurable aggregation²¹⁰. Additionally, at these higher-than-physiological concentrations, some ALS-associated SOD1 mutants, such as the G93D variant, show *in vitro* aggregation kinetics that are comparable to or slower than the kinetics of the WT protein.²¹⁰ This suggests that loss of function of the protein clearance machinery may not be sufficient to explain fully the onset of misfolding and aggregation, and that some additional, active process may be needed to promote mutant SOD1 misfolding and aggregation in ALS patient motor neurons.

The oxidative damage hypothesis offers a potential explanation for the late onset of motor neuron-specific misfolding of otherwise aggregation-resistant mutant SOD1. The hypothesis states that SOD1's normal role as a free radical scavenger puts it at risk of oxidative damage, which can trigger misfolding primed by mutation. According to this hypothesis, ALS-causing mutations could have the common property, not of promoting global SOD1 unfolding, but of priming SOD1 to populate aggregation-competent native-like misfolded states in response to denaturational stresses or covalent damage—states that the WT protein resists populating in response to the same stresses or damage.^{63,112,211,212} Elevated oxidative stress in disease-affected tissues, partially as a result of mitochondrial malfunction, is a known hallmark of ALS;^{89,213–216} furthermore, oxidative modifications to proteins, including SOD1, have been reported in mouse ALS models.^{213,217} This hypothesis is attractive, since, if it is correct, one would expect SOD1 to be most prone to oxidation-induced misfolding and aggregation in cells in which its half-life is longest (i.e. in which it has the greatest lifetime risk of being damaged). Since motor neurons are some of the physically longest cells in the body, with axons up to 1 m long, the slow transport processes that carry SOD1 from the cell body to the end of the axon necessitate an SOD1 half-life on the order of hundreds of days²¹²—much longer than in most cell types, which often have cellular lifespans shorter than this. Greater propensity to misfold and aggregate would also be expected in highly metabolically active cells in which the mitochondria produce more free radical species. Motor neurons, which use enormous amounts of energy to maintain their Na⁺ and K⁺ gradients, are an excellent example.^{90,218} Finally, this hypothesis provides a possible explanation for the late onset of disease, for although the mutant protein is present from birth, the oxidative stress that promotes misfolding and aggregation increases with age.^{218,219} Additionally, slow accumulation of oxidatively damaged SOD1 may result in a very long latency period before a sufficient concentration of misfolded protein is reached for aggregation-competent nuclei to form. The misfolding processes themselves can also have extraordinarily long half-lives.²²⁰

Many hypotheses have been advanced as to the shared feature of ALS-causing SOD1 mutations, and many of these have been proven not to hold true for all ALS-causing mutations. Declaring a new “common denominator” for SOD1 mutations causing ALS is therefore risky. That being said, recent *in vitro* studies (discussed in greater detail below) into mechanisms of SOD1 misfolding resulting from oxidative damage have shown that this damage triggers non-amyloid aggregation very similar to that seen in ALS patient tissues, and that the subset of ALS-associated SOD1 mutants that were tested had greater propensity to misfold and aggregate in

response to oxidative stress than did the WT protein.^{211,212,220} This suggests that the oxidative damage hypothesis is an hypothesis worthy of further study.

Studies of SOD1 unfolding and misfolding pathways

The development of an effective therapy for SOD1-associated fALS must target the underlying mechanisms of disease: SOD1 misfolding itself. One possible strategy involves stabilization of the native state to prevent formation of non-native, misfolded species. This has been a major strategy pursued for preventing the misfolding and aggregation of transthyretin (TTR), a plasma protein implicated in certain non-neurodegenerative amyloidoses. Analogues of thyroxine, the natural ligand of TTR, can bind selectively to the TTR native state, preventing the disruption of tertiary and quaternary structure that precedes aggregation.^{221–224} In the past, the application of this strategy to SOD1 has also been explored, and computational docking studies have produced a small number of compounds that were able to bind to the SOD1 native state and hinder SOD1 aggregation *in vitro*.²²⁵ However, natively-folded SOD1 is present throughout the body at high concentration, so that large amounts of a therapeutic agent able to bind this species would be needed if the strategy of stabilizing the native state were adopted, and much of the administered drug would bind uselessly to properly-folded SOD1 in tissues unaffected by disease. Sub-stoichiometric doses could be used, however, if specific therapeutic agents could be developed to bind surfaces uniquely exposed in disease-associated SOD1 misfolded states, and which could either stabilize misfolding intermediates upstream of the cytotoxic species or disrupt aberrant protein-protein interactions in which toxic misfolded species participate. Already, this strategy has proven effective *in vitro* for preventing TTR misfolding and aggregation. An antibody capable of recognizing a surface normally buried in the TTR tetramer, but which can become exposed as a result of changes in quaternary structure that occur during the misfolding and aggregation cascade, showed dramatic ability to prevent TTR aggregation, even at sub-stoichiometric concentrations.²²⁶ While there would be additional challenges associated with applying a similar strategy to a protein like SOD1, located within motor neurons (instead of free-floating in the bloodstream), the necessary first step in pursuing this strategy relies on careful characterization of the disease-relevant misfolded species. In the case of TTR, studies of the TTR misfolding mechanism had identified a tetramer dissociation step in the aggregation cascade, and this knowledge led to the identification of a disease-specific TTR epitope for antibody generation.^{226–229}

Given detailed characterization of misfolded species populated on the SOD1 unfolding or misfolding

pathways, it should be possible to identify good disease-associated targets for design of therapeutic agents to treat SOD1-associated fALS. In many cases, partially-structured species that are populated under conditions of steady-state equilibrium *in vivo* may be transiently populated *in vitro*, necessitating kinetic analysis to identify these species. Early kinetic studies of apo-SOD1 folding behavior used time-resolved tryptophan fluorescence or CD measurements to monitor conformational changes.^{163,177,230–233} These revealed complicated kinetics that were not consistent with a simple two-state folding transition, leading several groups to propose a three-state folding model in which folding of the SOD1 monomer precedes dimer association.^{177,230–233} This finding was also consistent with various equilibrium studies.^{234,235} Two studies that examined holo-SOD1 also proposed a three-state folding model, this one involving a metal-loaded, largely folded, monomeric intermediate.^{163,236} However, without direct measurements of Cu and Zn binding, it was not possible to produce a more detailed model describing the role that the many different possible partially metal-loaded states could play.

In 2008, our laboratory published a study of the kinetics of WT SOD1 unfolding, using guanidine HCl as a denaturant.¹⁶² This study used multiple read-outs reporting on different aspects of the SOD1 unfolding process, including an assay developed to provide direct measurement of release of bound Cu and Zn. This permitted more detailed characterization of unfolding intermediates than was previously possible, and allowed the intermediate described in previous studies^{163,177,230–236} to be subdivided into four distinct species with discernible metal occupancies. These experiments indicated that the WT protein unfolds primarily by way of a Zn-deficient but largely folded monomeric intermediate, but also revealed a minority pathway involving Cu-deficient intermediates with less residual structure.¹⁶² These pathways were confirmed by independent means by the Meiering group in 2009 in a study that was also able to examine the SOD1 refolding mechanism.²³⁷

Applying the techniques that we had developed to examine WT SOD1 to probe alterations in the SOD1 unfolding mechanism caused by ALS-associated mutations, our laboratory subsequently found that all of the ALS-associated mutations tested increase the propensity for SOD1 to unfold by way of the minority pathway that we had identified previously, populating more unstructured, Cu-deficient misfolded states.²³⁸ Beyond examining the SOD1 unfolding pathway using strongly denaturing conditions, we sought to establish conditions *in vitro* that could mimic the oxidative damage-promoting conditions which are hypothesized to trigger SOD1 misfolding and aggregation primed by mutation *in vivo*. This we accomplished through the use of hydrogen peroxide, a product of the dismutation reaction catalyzed by

SOD1, to drive the reaction backwards and to generate damaging free radical species at the SOD1 active site.²²⁰ Applying the previously-developed spectroscopic techniques, in combination with time-resolved NMR spectroscopy, to the examination of the slow misfolding triggered by the action of these free radical species, we were able to identify a long-lived, mildly misfolded early intermediate in the misfolding and aggregation cascade.²²⁰ This species lies upstream of many species with structures that are considerably more disrupted, particularly in the case of ALS-associated mutant SOD1, as assessed by endpoint species' capacity to bind conformation-specific antibodies SEDI and USOD, which were raised against epitopes that are buried in natively-folded SOD1 but exposed in structurally-disrupted SOD1.^{206,220} Stabilization of this early intermediate would slow the accumulation of the more drastically misfolded species most likely to be responsible for disease pathology, making the early intermediate an attractive candidate as a therapeutic target.

In our study, many different techniques yielding signals reporting on different structural changes were employed to examine SOD1 misfolding, revealing different degrees of structural rearrangement in different parts of the misfolded protein. In particular, fluorescence measurements indicated that the chemical environment of W32, located on β -strand 3 on the face of the β -barrel opposite to the active site, remains more or less unchanged despite major structural changes in the vicinity of the active site.²²⁰ NMR studies of the dynamics of apo-SOD1 carried out by Banci *et al.* have similarly revealed greater dynamic fluctuations in β -strands 4, 5, 7, and 8, forming the face of the β -barrel nearest the active site, than in β -strands 1, 2, 3, and 6, forming the distal face.²³⁹ Computational predictions by the Dokholyan group have also suggested that partial unfolding of one half of the β -barrel is possible, and that the nucleus for SOD1 aggregation could be the remaining, structured β -strands 1, 2, and 3 on the face distal to the active site, as well as the flanking strands 6 and 8.²⁴⁰ Although computational predictions, particularly of proteins with very slow misfolding kinetics, should be viewed with skepticism in isolation, taken together, these works make it possible to produce a crude map of the hypothetical residual native-like structure in misfolded SOD1. Figure 3 shows a qualitative map of the degree of conformational alteration from the native state as a result of oxidation-induced misfolding, with the most disrupted regions (primarily the metal binding loops and the β -barrel face proximal to the active site) shown in red and the regions of residual native-like structure (primarily the β -barrel face farthest from the active site) shown in green. Interestingly, there has now been a report of an ALS-causing SOD1 frameshift mutation that produces an early-onset ALS phenotype. The frameshift produces a severely truncated 35-amino acid product leaving only β -strands 1 and 2 intact and replacing part

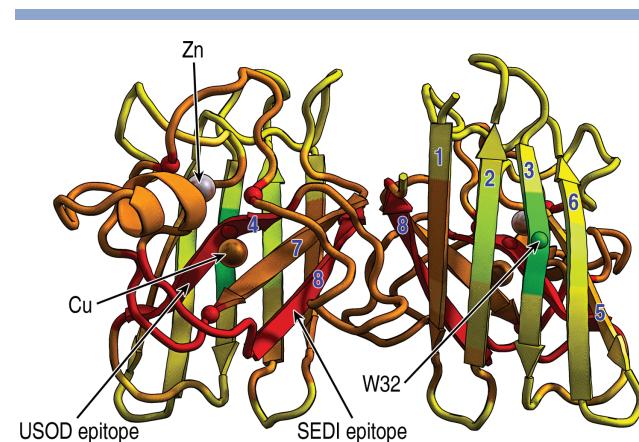


Figure 3

Rough, qualitative map of the degree of structural disruption to various regions of SOD1 as a result of oxidative damage-induced misfolding. A cartoon representation of PDB structure 1PU0 was generated and coloured in PyMOL 0.99rc6, and rendered with Maya 2012 and mental ray. Regions of greatest alteration are shown in red, regions of moderate alteration, in yellow, and regions of greatest retained native-like structure, in green. β -strands are numbered in blue. Circular dichroism (CD) measurements indicate that considerable secondary structure changes must occur as SOD1 misfolds, suggesting that much of the SOD1 β -barrel is affected.²²⁰ Nevertheless, tryptophan fluorescence studies have shown that the vicinity of W32 (shown as a green sphere), on the face of the SOD1 β -barrel opposite the active site, is relatively unaffected by the conformational changes that occur as SOD1 misfolds in response to oxidative damage.²²⁰ In contrast, NMR studies have revealed major changes to the chemical environments of active-site residues H46, H63, H71, and H120 (shown as red spheres).²²⁰ Exposure of the SEDI and USOD epitopes (at the dimer interface and under the active site, respectively) also occurs as a result of oxidative damage.²²⁰ Overall, the face of the protein that includes the active site appears to show more conformational disruption than the opposite face, which includes W32—a finding consistent with NMR studies of structural flexibility of apo-SOD1,²³⁹ and with computational predictions.²⁴⁰

of strand 3 with a different sequence before reaching a premature stop codon.²⁴¹ Whether this polypeptide is capable of achieving some structure in isolation and of nucleating aggregation by the same mechanism as full-length misfolded SOD1 has not been examined experimentally. If so, this region of the protein—contained within the region possessing native-like structure in Figure 3—would represent the minimum necessary sequence for the aggregation mechanism.

CONCLUDING REMARKS

The efforts summarized above to model the molecular details of a possible mechanism by which SOD1 misfolds, and to characterize the pre-toxic and toxic species on the misfolding pathway, have provided tantalizing glimpses of possible targets for rational drug design. Successful design efforts will necessarily rely on more detailed structural characterization of misfolded states, however—a difficult experimental problem that structural biochemistry has only begun to tackle. Nevertheless, it is hoped that

targeting early species on the disease-associated misfolding pathway with specific agents able to hinder production of downstream, toxic species will one day prove to be an effective therapeutic strategy for treating SOD1-associated ALS. Although studies probing the misfolding mechanisms of other aggregation-prone proteins implicated in other forms of ALS are currently at an earlier stage, a similar strategy can be envisaged for developing treatments for these forms of the disease as well.

ALS is distinct from other late-onset neurodegenerative diseases in that it involves the formation of non-amyloid, rather than amyloid, aggregates.^{111,112} Solid-state NMR studies of amyloid fibrils have revealed that these fibrils typically involve polypeptides in distinctly non-native states, in which backbone-backbone contacts are made between different subunits in elongated β -strand conformations.^{99–101} In contrast, the misfolding processes that give rise to aggregation-competent SOD1 in SOD1-associated ALS seem to preserve considerable amounts of native-like structure in the species produced.^{220,240} This raises the possibility that retention of native-like structure may distinguish the misfolding and aggregation cascades that underlie non-amyloid diseases from those underlying the amyloid diseases. If this holds true in cases of ALS not caused by SOD1 mutations, this would set ALS apart from the other late-onset neurodegenerative diseases in the taxonomy of disease, and could have important consequences for the development of general therapeutic strategies—in particular, for the question of whether we should be seeking broad therapeutics able to block misfolding and aggregation mechanisms common to all late-onset neurodegenerative diseases, or more specific drugs able to target each disease individually. We find ourselves at a point at which the uncharted territory is still far vaster than the charted, however. In order to answer questions about the relationships between the late-onset neurodegenerative diseases, and even of the relationships between the diverse forms of each disease, it will be necessary to elucidate the misfolding and aggregation mechanisms of many more of the causative proteins.

REFERENCES

- Oeppen J, Vaupel JW. Broken limits to life expectancy. *Science* 2002;296:1029–1031.
- World Health Statistics 2011. Geneva, Switzerland: WHO Department of Health Statistics and Informatics; 2011. pp 170.
- Moss WJ, Griffin DE. Measles. *Lancet* 2012;379:153–164.
- Measles vaccines: WHO position paper. *Wkly Epidemiol Rec* 2009;84:349–360.
- Schreiber A, Hershman G. Non-HIV viral infections of the salivary glands. *Oral Maxillofac Surg Clin N Am* 2009;21:331–338.
- Hviid A, Rubin S, Muhlemann K. Mumps. *Lancet* 2008;371:932–944.
- Nathanson N, Kew OM. From emergence to eradication: the epidemiology of poliomyelitis deconstructed. *Am J Epidemiol* 2010;172:1213–1229.
- Lane JM. Mass vaccination and surveillance/containment in the eradication of smallpox. *Curr Topics Microbiol Immunol* 2006;304:17–29.
- Vazquez M, LaRussa PS, Gershon AA, Steinberg SP, Freudigman K, Shapiro ED. The effectiveness of the varicella vaccine in clinical practice. *N Engl J Med* 2001;344:955–960.
- Marin M, Zhang JX, Seward JF. Near elimination of varicella deaths in the US after implementation of the vaccination program. *Pediatrics* 2011;128:214–220.
- Clark NM, Lynch JP, III. Influenza: epidemiology, clinical features, therapy, and prevention. *Semin Respir Crit Care Med* 2011;32:373–392.
- Friede M, Palkonyay L, Alfonso C, Pervikov Y, Torelli G, Wood D, Kiely MP. WHO initiative to increase global and equitable access to influenza vaccine in the event of a pandemic: supporting developing country production capacity through technology transfer. *Vaccine* 2011;29(Suppl 1):A2–A7.
- Merle CS, Cunha SS, Rodrigues LC. BCG vaccination and leprosy protection: review of current evidence and status of BCG in leprosy control. *Expert Rev Vaccines* 2010;9:209–222.
- Rodrigues LC, Lockwood D. Leprosy now: epidemiology, progress, challenges, and research gaps. *Lancet Infect Dis* 2011;11:464–470.
- Clemens JD. Vaccines in the time of cholera. *Proc Natl Acad Sci USA* 2011;108:8529–8530.
- Sur D, Lopez AL, Kanungo S, Paisley A, Manna B, Ali M, Niyogi SK, Park JK, Sarkar B, Puri MK, Kim DR, Deen JL, Holmgren J, Carbis R, Rao R, Nguyen TV, Donner A, Ganguly NK, Nair GB, Bhattacharya SK, Clemens JD. Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. *Lancet* 2009;374:1694–1702.
- Charles RC, Ryan ET. Cholera in the 21st century. *Curr Opin Infect Dis* 2011;24:472–477.
- Collins JJ. The contribution of medical measures to the decline of mortality from respiratory tuberculosis: an age-period-cohort model. *Demography* 1982;19:409–427.
- Onozaki I, Ravaglione M. Stopping tuberculosis in the 21st century: goals and strategies. *Respirology* 2010;15:32–43.
- Atre SR, Rangan SG, Shetty VP, Gaikwad N, Mistry NF. Perceptions, health seeking behaviour and access to diagnosis and treatment initiation among previously undetected leprosy cases in rural Maharashtra, India. *Lepr Rev* 2011;82:222–234.
- Banta HD. Global issues on the agenda at the World Health Assembly: discussion of HIV/AIDS, leprosy, access to drugs. *JAMA* 2001;286:29–30.
- Keshavjee S, Farmer PE. Time to put boots on the ground: making universal access to MDR-TB treatment a reality. *Int J Tuberc Lung Dis* 2010;14:1222–1225.
- Park PH, Magut C, Gardner A, O’Yiengo D O, Kamle L, Langat BK, Buziba NG, Carter EJ. Increasing access to the MDR-TB surveillance programme through a collaborative model in western Kenya. *Tropical Med Int Health* 2012;17:374–379.
- Lienhardt C, Vernon A, Ravaglione MC. New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. *Curr Opin Pulm Med* 2010;16:186–193.
- Price DL. Aging of the brain and dementia of the Alzheimer type. In: Kandel ER, Schwartz JH, Jessell TM, editors. *Principles of neural science*, 4th ed. New York: McGraw-Hill; 2000. pp 1149–1161.
- Leading Causes of Death in Canada, 2008: StatsCan publication 84-215-X, Vol. 2011: Statistics Canada; 2011.
- Leading Causes of Death in Canada, 2009: StatsCan publication 84-215-XWE, Vol. 2012: Statistics Canada; 2012.
- Murphy SLX, J., Kochanek KD. Deaths: preliminary data for 2010. National vital statistics reports: from the Centers for Disease Control and Prevention, National Center for Health Statistics. *Natl Vital Stat Syst* 2012;60:1–68.

29. Minino AM, Murphy SL, Xu J, Kochanek KD. Deaths: final data for 2008. National vital statistics reports: from the Centers for Disease Control and Prevention, National Center for Health Statistics. *Natl Vital Stat Syst* 2011;59:1–126.
30. Reddel RR. A reassessment of the telomere hypothesis of senescence. *Bioessays* 1998;20:977–984.
31. Hamill RW, Marquesberry WR, McDaniel K, Coleman PD. Characterization of brain samples in studies of aging, Alzheimer's, and other neurodegenerative diseases. *Neurobiol Aging* 1993;14:539–545.
32. Novak MJ, Tabrizi SJ. Huntington's disease: clinical presentation and treatment. *Int Rev Neurobiol* 2011;98:297–323.
33. Saxena S, Caroni P. Selective neuronal vulnerability in neurodegenerative diseases: from stressor thresholds to degeneration. *Neuron* 2011;71:35–48.
34. Ince PG, Lowe J, Shaw PJ. Amyotrophic lateral sclerosis: current issues in classification, pathogenesis and molecular pathology. *Neuropathol Appl Neurobiol* 1998;24:104–117.
35. Mitchell JD, Borasio GD. Amyotrophic lateral sclerosis. *Lancet* 2007;369:2031–2041.
36. Heron M, Hoyert DL, Murphy SL, Xu J, Kochanek KD, Tejada-Vera B. Deaths: final data for 2006. National vital statistics reports: from the Centers for Disease Control and Prevention, National Center for Health Statistics. *Natl Vital Stat Syst* 2009;57:1–134.
37. Lees AJ. L-dopa treatment and Parkinson's disease. *Q J Med* 1986;59:535–547.
38. Foltynie T, Michell AW, Barker RA. Parkinson's disease: what is it? what causes it? and how can it be cured? In: Smith HJ, Simons C, Sewell RDE, editors. *Protein misfolding in neurodegenerative diseases*. Boca Raton, FL: CRC Press; 2008. pp 381–414.
39. Small DH. Acetylcholinesterase inhibitors for the treatment of dementia in Alzheimer's disease: do we need new inhibitors? *Expert Opin Emerg Drugs* 2005;10:817–825.
40. Cheah BC, Vucic S, Krishnan AV, Kiernan MC. Riluzole, neuroprotection and amyotrophic lateral sclerosis. *Curr Med Chem* 2010;17:1942–1959.
41. Molinuevo JL, Llado A, Rami L. Memantine: targeting glutamate excitotoxicity in Alzheimer's disease and other dementias. *Am J Alzheimers Dis Other Demen* 2005;20:77–85.
42. Bellingham MC. A review of the neural mechanisms of action and clinical efficiency of riluzole in treating amyotrophic lateral sclerosis: what have we learned in the last decade? *CNS Neurosci Therap* 2011;17:4–31.
43. Mitchell JD, O'Brien M R, Joshi M. Audit of outcomes in motor neuron disease (MND) patients treated with riluzole. *Amyotroph Lateral Scler* 2006;7:67–71.
44. Herrmann N, Chau SA, Kircanski I, Lanctot KL. Current and emerging drug treatment options for Alzheimer's disease: a systematic review. *Drugs* 2011;71:2031–2065.
45. Schneider LS, Dagerman KS, Higgins JP, McShane R. Lack of evidence for the efficacy of memantine in mild Alzheimer disease. *Arch Neurol* 2011;68:991–998.
46. Petersen RC. New clinical criteria for the Alzheimer's disease spectrum. *Minnesota Med* 2012;95:42–45.
47. Albanese A. Diagnostic criteria for Parkinson's disease. *Neurol Sci* 2003;24(Suppl 1):S23–S26.
48. Blonder LX, Slevin JT. Emotional dysfunction in Parkinson's disease. *Behav Neurol* 2011;24:201–217.
49. Brown K, Mastrianni JA. The prion diseases. *J Geriatr Psychiatry Neurol* 2010;23:277–298.
50. Prusiner SB. The prion diseases. *Brain Pathol* 1998;8:499–513.
51. Barash JA. Clinical features of sporadic fatal insomnia. *Rev Neurol Dis* 2009;6:E87–E93.
52. Lugaresi E, Tobler I, Gambetti P, Montagna P. The pathophysiology of fatal familial insomnia. *Brain Pathol* 1998;8:521–526.
53. Medori R, Tritschler HJ, LeBlanc A, Villare F, Manetto V, Chen HY, Xue R, Leal S, Montagna P, Cortelli P, Tinuper P, Avoni P, Mochi M, Baruzzi A, Hauw JJ, Ott J, Lugaresi E, Autilio-Gambetti L, Gambetti P. Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. *N Engl J Med* 1992;326:444–449.
54. Reiner A, Dragatsis I, Dietrich P. Genetics and neuropathology of Huntington's disease. *Int Rev Neurobiol* 2011;98:325–372.
55. Goldman SM, Tanner CM, Oakes D, Bhudhikanok GS, Gupta A, Langston JW. Head injury and Parkinson's disease risk in twins. *Ann Neurol* 2006;60:65–72.
56. Lai BC, Marion SA, Teschke K, Tsui JK. Occupational and environmental risk factors for Parkinson's disease. *Parkinsonism Relat Disord* 2002;8:297–309.
57. Liu B, Gao HM, Hong JS. Parkinson's disease and exposure to infectious agents and pesticides and the occurrence of brain injuries: role of neuroinflammation. *Environ Health Perspect* 2003;111:1065–1073.
58. Lesage S, Brice A. Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Hum Mol Genet* 2009;18:R48–R59.
59. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenoos ES, Chandrasekharappa S, Athanasiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997;276:2045–2047.
60. Rogeava E. The solved and unsolved mysteries of the genetics of early-onset Alzheimer's disease. *Neuromol Med* 2002;2:1–10.
61. Sherrington R, Rogaev EI, Liang Y, Rogeava EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Perkicak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St George-Hyslop PH. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 1995;375:754–760.
62. Majoor-Krakauer D, Willems PJ, Hofman A. Genetic epidemiology of amyotrophic lateral sclerosis. *Clin Genet* 2003;63:83–101.
63. Rakshit R, Chakrabarty A. Structure, folding, and misfolding of Cu, Zn superoxide dismutase in amyotrophic lateral sclerosis. *Biochim Biophys Acta* 2006;1762:1025–1037.
64. Prusiner SB, DeArmond SJ. Molecular biology and pathology of scrapie and the prion diseases of humans. *Brain Pathol* 1991;1:297–310.
65. Prusiner SB. Scrapie prions. *Annu Rev Microbiol* 1989;43:345–374.
66. Dickson DW, Braak H, Duda JE, Duyckaerts C, Gasser T, Halliday GM, Hardy J, Leverenz JB, Del Tredici K, Wszolek ZK, Litvan I. Neuropathological assessment of Parkinson's disease: refining the diagnostic criteria. *Lancet Neurol* 2009;8:1150–1157.
67. Rahimi F, Bitan G. Overview of fibrillar and oligomeric assemblies of amyloidogenic proteins. In: Rahimi F, Bitan G, editors. *Non-fibrillar amyloidogenic protein assemblies—common cytotoxins underlying degenerative diseases*. Dordrecht, The Netherlands: Springer; 2012. pp 1–36.
68. Munch C, Bertolotti A. Propagation of the prion phenomenon: beyond the seeding principle. *J Mol Biol* 2012;421:491–498.
69. Bertram L, Tanzi RE. The genetic epidemiology of neurodegenerative disease. *J Clin Investig* 2005;115:1449–1457.
70. Bates G. Huntingtin aggregation and toxicity in Huntington's disease. *Lancet* 2003;361:1642–1644.
71. Ignatova Z, Gerasch LM. Extended polyglutamine tracts cause aggregation and structural perturbation of an adjacent beta barrel protein. *J Biol Chem* 2006;281:12959–12967.
72. Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St George Hyslop P, Selkoe DJ. Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat Med* 1997;3:67–72.

73. Bateman DA, Chakrabarty A. Interactions of Alzheimer amyloid peptides with cultured cells and brain tissue, and their biological consequences. *Biopolymers* 2004;76:4–14.
74. Gorman PM, Chakrabarty A. Alzheimer beta-amyloid peptides: structures of amyloid fibrils and alternate aggregation products. *Bio-polymers* 2001;60:381–394.
75. Morales R, Duran-Aniotz CA, Soto C. Role of prion protein oligomers in the pathogenesis of transmissible spongiform encephalopathies. In: Rahimi F, Bitan G, editors. Non-fibrillar Amyloidogenic Protein Assemblies – Common Cytotoxins Underlying Degenerative Diseases. Dordrecht, The Netherlands: Springer; 2012. pp 319–335.
76. Fawzi NL, Phillips AH, Ruscio JZ, Doucleff M, Wemmer DE, Head-Gordon T. Structure and dynamics of the Abeta(21–30) peptide from the interplay of NMR experiments and molecular simulations. *J Am Chem Soc* 2008;130:6145–6158.
77. Vivekanandan S, Brender JR, Lee SY, Ramamoorthy A. A partially folded structure of amyloid-beta(1–40) in an aqueous environment. *Biochem Biophys Res Commun* 2011;411:312–316.
78. Dobson CM. Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol* 2004;15:3–16.
79. Naeem A, Fazili NA. Defective protein folding and aggregation as the basis of neurodegenerative diseases: the darker aspect of proteins. *Cell Biochem Biophys* 2011;61:237–250.
80. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* 2006;443:780–786.
81. Ying Z, Wang H, Wang G. The Ubiquitin Proteasome System as a Potential Target for the Treatment of Neurodegenerative Diseases. *Current Pharma Design*, in press.
82. Meredith SC. Protein denaturation and aggregation: cellular responses to denatured and aggregated proteins. *Ann NY Acad Sci* 2005;1066:181–221.
83. Milanesi L, Sheynis T, Xue WF, Orlova EV, Hellewell AL, Jelinek R, Hewitt EW, Radford SE, Saibil HR. Direct three-dimensional visualization of membrane disruption by amyloid fibrils. *Proc Natl Acad Sci USA* 2012;109:20455–20460.
84. Williams TL, Johnson BR, Urbanc B, Jenkins AT, Connell SD, Serpell LC. Abeta42 oligomers, but not fibrils, simultaneously bind to and cause damage to ganglioside-containing lipid membranes. *Biochem J* 2011;439:67–77.
85. Chung J, Yang H, de Beus MD, Ryu CY, Cho K, Colon W. Cu/Zn superoxide dismutase can form pore-like structures. *Biochem Biophys Res Commun* 2003;312:873–876.
86. Rambold AS, Miesbauer M, Rapaport D, Bartke T, Baier M, Winklhofer KF, Tatzelt J. Association of Bcl-2 with misfolded prion protein is linked to the toxic potential of cytosolic PrP. *Mol Biol Cell* 2006;17:3356–3368.
87. Chai Y, Shao J, Miller VM, Williams A, Paulson HL. Live-cell imaging reveals divergent intracellular dynamics of polyglutamine disease proteins and supports a sequestration model of pathogenesis. *Proc Natl Acad Sci USA* 2002;99:9310–9315.
88. Faes L, Callewaert G. Mitochondrial dysfunction in familial amyotrophic lateral sclerosis. *J Bioenerg Biomembr* 2011;43:587–592.
89. Shi P, Wei Y, Zhang J, Gal J, Zhu H. Mitochondrial dysfunction is a converging point of multiple pathological pathways in amyotrophic lateral sclerosis. *J Alzheimers Dis* 2010;20(Suppl 2):S311–S324.
90. von Lewinski F, Keller BU. Ca²⁺, mitochondria and selective motor neuron vulnerability: implications for ALS. *Trends Neurosci* 2005;28:494–500.
91. Sipe JD. Amyloidosis. *Annu Rev Biochem* 1992;61:947–975.
92. Chen S, Berthelier V, Hamilton JB, O’Nuallain B, Wetzel R. Amyloid-like features of polyglutamine aggregates and their assembly kinetics. *Biochemistry* 2002;41:7391–7399.
93. Westermark P, Benson MD, Buxbaum JN, Cohen AS, Frangione B, Ikeda S, Masters CL, Merlini G, Saraiva MJ, Sipe JD. Amyloid: toward terminology clarification. Report from the Nomenclature Committee of the International Society of Amyloidosis. *Amyloid* 2005;12:1–4.
94. Kyle RA. Amyloidosis: a convoluted story. *Br J Haematol* 2001;114:529–538.
95. Comenzo RL. Systemic immunoglobulin light-chain amyloidosis. *Clin Lymphoma Myeloma* 2006;7:182–185.
96. Stathopoulos PB, Scholz GA, Hwang YM, Rumfeldt JA, Lepock JR, Meiering EM. Sonication of proteins causes formation of aggregates that resemble amyloid. *Protein Sci* 2004;13:3017–3027.
97. Chiti F, Webster P, Taddei N, Clark A, Stefani M, Ramponi G, Dobson CM. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. *Proc Natl Acad Sci USA* 1999;96:3590–3594.
98. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem* 2006;75:333–366.
99. Antzutkin ON, Leapman RD, Balbach JJ, Tycko R. Supramolecular structural constraints on Alzheimer’s beta-amyloid fibrils from electron microscopy and solid-state nuclear magnetic resonance. *Biochemistry* 2002;41:15436–15450.
100. Benzinger TL, Gregory DM, Burkoth TS, Miller-Auer H, Lynn DG, Botto RE, Meredith SC. Propagating structure of Alzheimer’s beta-amyloid(10–35) is parallel beta-sheet with residues in exact register. *Proc Natl Acad Sci USA* 1998;95:13407–13412.
101. Tycko R. Solid-state NMR studies of amyloid fibril structure. *Annu Rev Phys Chem* 2011;62:279–299.
102. Feiner HD. Pathology of dysproteinemia: light chain amyloidosis, non-amyloid immunoglobulin deposition disease, cryoglobulinemia syndromes, and macroglobulinemia of Waldenstrom. *Hum Pathol* 1988;19:1255–1272.
103. Polski JM, Galvin N, Salinas-Madrigal L. Non-amyloid fibrils in heavy chain deposition disease. *Kidney Int* 1999;56:1601–1602.
104. Dickson DW. Discovery of new lesions in neurodegenerative diseases with monoclonal antibody techniques: is there a non-amyloid precursor to senile plaques? *Am J Pathol* 1997;151:7–11.
105. Lemere CA, Grenfell TJ, Selkoe DJ. The AMY antigen co-occurs with abeta and follows its deposition in the amyloid plaques of Alzheimer’s disease and down syndrome. *Am J Pathol* 1999;155:29–37.
106. Schmidt ML, Lee VM, Forman M, Chiu TS, Trojanowski JQ. Monoclonal antibodies to a 100-kd protein reveal abundant A beta-negative plaques throughout gray matter of Alzheimer’s disease brains. *Am J Pathol* 1997;151:69–80.
107. Chattopadhyay M, Durazo A, Sohn SH, Strong CD, Gralla EB, Whitelegge JP, Valentine JS. Initiation and elongation in fibrillation of ALS-linked superoxide dismutase. *Proc Natl Acad Sci USA* 2008;105:18663–18668.
108. Chattopadhyay M, Valentine JS. Aggregation of copper-zinc superoxide dismutase in familial and sporadic ALS. *Antioxid Redox Signal* 2009;11:1603–1614.
109. Rousseau F, Schymkowitz J, Oliveberg M. ALS precursor finally shaken into fibrils. *Proc Natl Acad Sci USA* 2008;105:18649–18650.
110. Fandrich M, Fletcher MA, Dobson CM. Amyloid fibrils from muscle myoglobin. *Nature* 2001;410:165–166.
111. Kerman A, Liu HN, Croul S, Bilbao J, Rogaeva E, Zinman L, Robertson J, Chakrabarty A. Amyotrophic lateral sclerosis is a non-amyloid disease in which extensive misfolding of SOD1 is unique to the familial form. *Acta Neuropathol* 2010;119:335–344.
112. Kerman A, Chakrabarty A. Protein misfolding and toxicity in amyotrophic lateral sclerosis. In: Rahimi F, Bitan G, editors. Non-fibrillar amyloidogenic protein assemblies—common cytotoxins underlying degenerative diseases. Dordrecht, The Netherlands: Springer; 2012. pp 257–288.
113. Kato S, Takikawa M, Nakashima K, Hirano A, Cleveland DW, Kusaka H, Shibata N, Kato M, Nakano I, Ohama E. New consensus research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 (SOD1) gene mutations: inclusions containing SOD1 in neurons and astrocytes.

- Amyotroph Lateral Scler Other Motor Neuron Disord 2000;1:163–184.
114. Arai T, Hasegawa M, Akiyama H, Ikeda K, Nonaka T, Mori H, Mann D, Tsuchiya K, Yoshida M, Hashizume Y, Oda T. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* 2006;351:602–611.
 115. Mackenzie IR, Bigio EH, Ince PG, Geser F, Neumann M, Cairns NJ, Kwong LK, Forman MS, Ravits J, Stewart H, Eisen A, McClusky L, Kretzschmar HA, Monoranu CM, Highley JR, Kirby J, Siddique T, Shaw PJ, Lee VM, Trojanowski JQ. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol* 2007;61:427–434.
 116. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM. Ubiquitininated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006;314:130–133.
 117. Tan CF, Eguchi H, Tagawa A, Onodera O, Iwasaki T, Tsujino A, Nishizawa M, Kakita A, Takahashi H. TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta Neuropathol* 2007;113:535–542.
 118. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuurra S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R, Kawakami H. Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 2010;465:223–226.
 119. Hortobagyi T, Troakes C, Nishimura AL, Vance C, van Swieten JC, Seelaar H, King A, Al-Sarraj S, Rogelj B, Shaw CE. Optineurin inclusions occur in a minority of TDP-43 positive ALS and FTLD-TDP cases and are rarely observed in other neurodegenerative disorders. *Acta Neuropathol* 2011;121:519–527.
 120. Ito H, Fujita K, Nakamura M, Wate R, Kaneko S, Sasaki S, Yamane K, Suzuki N, Aoki M, Shibata N, Togashi S, Kawata A, Mochizuki Y, Mizutani T, Maruyama H, Hirano A, Takahashi R, Kawakami H, Kusaka H. Optineurin is co-localized with FUS in basophilic inclusions of ALS with FUS mutation and in basophilic inclusion body disease. *Acta Neuropathol* 2011;121:555–557.
 121. van Blitterswijk M, van Vught PW, van Es MA, Schelhaas HJ, van der Kooi AJ, de Visser M, Veldink JH, van den Berg LH. Novel optineurin mutations in sporadic amyotrophic lateral sclerosis patients. *Neurobiol Aging* 2012;33:1016.e1–1016.e7.
 122. Kwiatkowski TJ, Jr, Bosco DA, Leclerc AL, Tamrazian E, Vandenburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, Valdmanis P, Rouleau GA, Hosler BA, Cortelli P, de Jong PJ, Yoshinaga Y, Haines JL, Pericak-Vance MA, Yan J, Ticicci N, Siddique T, McKenna-Yasek D, Sapp PC, Horvitz HR, Landers JE, Brown RH, Jr. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 2009;323:1205–1208.
 123. Vance C, Rogelj B, Hortobagyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, Ganeshalingam J, Williams KL, Tripathi V, Al-Sarraj S, Al-Chalabi A, Leigh PN, Blair IP, Nicholson G, de Belleroche J, Gallo JM, Miller CC, Shaw CE. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 2009;323:1208–1211.
 124. Udan M, Baloh RH. Implications of the prion-related Q/N domains in TDP-43 and FUS. *Prion* 2011;5:1–5.
 125. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, Rahmani Z, Kriuszus A, McKenna-Yasek D, Cayabyab A, Gaston SM, Berger R, Tanzi RE, Halperin JJ, Herzfeldt B, Van Den Bergh R, Hung W, Bird T, Deng G, Mulder DW, Smyth C, Laing NG, Soriano E, Pericak-Vance MA, Haines J, Rouleau GA, Gusella JS, Horvitz HR, Brown RH, Jr. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993;362:59–62.
 126. Brujin LI, Housewright MK, Kato S, Anderson KL, Anderson SD, Ohama E, Reame AG, Scott RW, Cleveland DW. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. *Science* 1998;281:1851–1854.
 127. Okado-Matsumoto A, Myint T, Fujii J, Taniguchi N. Gain in functions of mutant Cu,Zn-superoxide dismutases as a causative factor in familial amyotrophic lateral sclerosis: less reactive oxidant formation but high spontaneous aggregation and precipitation. *Free Radic Res* 2000;33:65–73.
 128. Robinson JL, Geser F, Stieber A, Umoh M, Kwong LK, Van Deerlin VM, Lee VM, Trojanowski JQ. TDP-43 skeins show properties of amyloid in a subset of ALS cases. *Acta Neuropathol* 2013;125:121–131.
 129. LeVine H, 3rd. Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci* 1993;2:404–410.
 130. Rowland LP. How amyotrophic lateral sclerosis got its name: the clinical-pathologic genius of Jean-Martin Charcot. *Arch Neurol* 2001;58:512–515.
 131. Charcot J-M. De la sclérose latérale amyotrophique: symptomatologie. *Le Progrès Méd* 1874;2:453–455.
 132. Goetz CG. Amyotrophic lateral sclerosis: early contributions of Jean-Martin Charcot. *Muscle Nerve* 2000;23:336–343.
 133. Lillo P, Savage S, Mioshi E, Kiernan MC, Hodges JR. Amyotrophic lateral sclerosis and frontotemporal dementia: a behavioural and cognitive continuum. *Amyotroph Lateral Scler* 2012;13:102–109.
 134. Ringholz GM, Greene SR. The relationship between amyotrophic lateral sclerosis and frontotemporal dementia. *Curr Neurol Neurosci Rep* 2006;6:387–392.
 135. Sanelli T, Robertson J, Chakrabarty A, Strong MJ. Amyotrophic lateral sclerosis (motor neuron disease). In: Smith HJ, Simons C, Sewell RDE, editors. *Protein misfolding in neurodegenerative diseases*. Boca Raton, FL: CRC Press; 2008. pp 479–514.
 136. Andersen PM, Al-Chalabi A. Clinical genetics of amyotrophic lateral sclerosis: what do we really know? *Nat Rev Neurol* 2011;7:603–615.
 137. Alonso A, Logroscino G, Jick SS, Hernan MA. Incidence and lifetime risk of motor neuron disease in the United Kingdom: a population-based study. *Eur J Neurol* 2009;16:745–751.
 138. Hardiman O, van den Berg LH, Kiernan MC. Clinical diagnosis and management of amyotrophic lateral sclerosis. *Nat Rev Neurol* 2011;7:639–649.
 139. Charcot J-M. Sclérose des cordons latéraux de la moelle épinière chez une femme hystérique atteinte de contracture permanente des quatre membres. *Bull Mem Soc Med Hop Paris* 1865:24–35.
 140. Charcot J-M. Deux cas d'atrophie musculaire progressive avec lésions de la substance grise et de faisceaux antérolatéraux de la moelle épinière. *Arch Physiol Norm Pathol* 1869;2:744–757.
 141. Ravits JM, La Spada AR. ALS motor phenotype heterogeneity, focality, and spread: deconstructing motor neuron degeneration. *Neurology* 2009;73:805–811.
 142. Kuwabara S, Yokota T. Propagation: prion-like mechanisms can explain spreading of motor neuronal death in amyotrophic lateral sclerosis? *J Neurol Neurosurg Psychiatry* 2011;82:1181–1182.
 143. Guest WC, Silverman JM, Pokrishevsky E, O'Neill MA, Grad LI, Cashman NR. Generalization of the prion hypothesis to other neurodegenerative diseases: an imperfect fit. *J Toxicol Environ Health A* 2011;74:1433–1459.
 144. Prusiner SB. Cell biology. A unifying role for prions in neurodegenerative diseases. *Science* 2012;336:1511–1513.
 145. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969;244:6049–6055.

146. ALSOD: the amyotrophic lateral sclerosis online genetics database. 2012. Accessed on 7 March 2012. Available from: <http://alsod.iop.kcl.ac.uk/index.aspx>.
147. Borchelt DR, Lee MK, Slunt HS, Guarnieri M, Xu ZS, Wong PC, Brown RH, Jr, Price DL, Sisodia SS, Cleveland DW. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc Natl Acad Sci USA* 1994;91:8292–8296.
148. Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH, Jr, Scott RW, Snider WD. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet* 1996;13:43–47.
149. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, Chen W, Zhai P, Sufit RL, Siddique T. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994;264:1772–1775.
150. Brujin LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 1997;18:327–338.
151. Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 1995;14:1105–1116.
152. Crow JP, Sampson JB, Zhuang Y, Thompson JA, Beckman JS. Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. *J Neurochem* 1997;69:1936–1944.
153. Estevez AG, Crow JP, Sampson JB, Reiter C, Zhuang Y, Richardson GJ, Tarpey MM, Barbeito L, Beckman JS. Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. *Science* 1999;286:2498–2500.
154. Wang J, Slunt H, Gonzales V, Fromholt D, Coonfield M, Copeland NG, Jenkins NA, Borchelt DR. Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature. *Hum Mol Genet* 2003;12:2753–2764.
155. DiDonato M, Craig L, Huff ME, Thayer MM, Cardoso RM, Kassmann CJ, Lo TP, Bruns CK, Powers ET, Kelly JW, Getzoff ED, Tainer JA. ALS mutants of human superoxide dismutase form fibrous aggregates via framework destabilization. *J Mol Biol* 2003;332:601–615.
156. Roberts VA, Fisher CL, Redford SM, McRee DE, Parge HE, Getzoff ED, Tainer JA. Mechanism and atomic structure of superoxide dismutase. *Free Radic Res Commun* 1991;12-13:269–278.
157. Richardson JS, Richardson DC, Thomas KA, Silverton EW, Davies DR. Similarity of three-dimensional structure between the immunoglobulin domain and the copper, zinc superoxide dismutase subunit. *J Mol Biol* 1976;102:221–235.
158. Stevens FJ. Homology versus analogy: possible evolutionary relationship of immunoglobulins, cupredoxins, and Cu, Zn-superoxide dismutase. *J Mol Recognit* 2008;21:20–29.
159. Tainer JA, Getzoff ED, Beem KM, Richardson JS, Richardson DC. Determination and analysis of the 2 A-structure of copper, zinc superoxide dismutase. *J Mol Biol* 1982;160:181–217.
160. Roberts BR, Tainer JA, Getzoff ED, Malencik DA, Anderson SR, Bomben VC, Meyers KR, Karplus PA, Beckman JS. Structural characterization of zinc-deficient human superoxide dismutase and implications for ALS. *J Mol Biol* 2007;373:877–890.
161. Banci L, Benedetto M, Bertini I, Del Conte R, Piccioli M, Viezzoli MS. Solution structure of reduced monomeric Q133M2 copper, zinc superoxide dismutase (SOD). Why is SOD a dimeric enzyme? *Biochemistry* 1998;37:11780–11791.
162. Mulligan VK, Kerman A, Ho S, Chakrabarty A. Denaturational stress induces formation of zinc-deficient monomers of Cu,Zn superoxide dismutase: implications for pathogenesis in amyotrophic lateral sclerosis. *J Mol Biol* 2008;383:424–436.
163. Rumfeldt JA, Stathopoulos PB, Chakrabarty A, Lepock JR, Meiering EM. Mechanism and thermodynamics of guanidinium chloride-induced denaturation of ALS-associated mutant Cu,Zn superoxide dismutases. *J Mol Biol* 2006;355:106–123.
164. Redler RL, Wilcox KC, Proctor EA, Fee L, Caplow M, Dokholyan NV. Glutathionylation at Cys-111 induces dissociation of wild type and FALS mutant SOD1 dimers. *Biochemistry* 2011;50:7057–7066.
165. Wilcox KC, Zhou L, Jordon JK, Huang Y, Yu Y, Redler RL, Chen X, Caplow M, Dokholyan NV. Modifications of superoxide dismutase (SOD1) in human erythrocytes: a possible role in amyotrophic lateral sclerosis. *J Biol Chem* 2009;284:13940–13947.
166. Calabrese L, Federici G, Bannister WH, Bannister JV, Rotilio G, Finazzi-Agro A. Labile sulfur in human Superoxide dismutase. *Eur J Biochem* 1975;56:305–309.
167. de Beus MD, Chung J, Colon W. Modification of cysteine 111 in Cu/Zn superoxide dismutase results in altered spectroscopic and biophysical properties. *Protein Sci* 2004;13:1347–1355.
168. You Z, Cao X, Taylor AB, Hart PJ, Levine RL. Characterization of a covalent polysulfane bridge in copper-zinc superoxide dismutase. *Biochemistry* 2010;49:1191–1198.
169. Mulligan VK, Hadley KC, Chakrabarty A. Analyzing complicated protein folding kinetics rapidly by analytical Laplace inversion using a Tikhonov regularization variant. *Anal Biochem* 2012;421:181–190.
170. Okado-Matsumoto A, Guan Z, Fridovich I. Modification of cysteine 111 in human Cu,Zn-superoxide dismutase. *Free Radic Biol Med* 2006;41:1837–1846.
171. Carrico RJ, Deutsch HF. Isolation of human hepatocuprein and cerebrocuprein. Their identity with erythrocuprein. *J Biol Chem* 1969;244:6087–6093.
172. Bannister WH, Dalgleish DG, Bannister JV, Wood EJ. Physicochemical and spectroscopic properties of human erythrocyte cupro-zinc protein (erythrocuprein). *Int J Biochem* 1972;3:560–568.
173. Lepock JR, Frey HE, Hallewell RA. Contribution of conformational stability and reversibility of unfolding to the increased thermostability of human and bovine superoxide dismutase mutated at free cysteines. *J Biol Chem* 1990;265:21612–21618.
174. Stathopoulos PB, Rumfeldt JA, Scholz GA, Irani RA, Frey HE, Hallewell RA, Lepock JR, Meiering EM. Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates in vitro. *Proc Natl Acad Sci USA* 2003;100:7021–7026.
175. Biliaderis CG, Weselake RJ, Petkau A, Friesen AD. A calorimetric study of human CuZn superoxide dismutase. *Biochem J* 1987;248:981–984.
176. Lepock JR. Measurement of protein stability and protein denaturation in cells using differential scanning calorimetry. *Methods* 2005;35:117–125.
177. Mei G, Rosato N, Silva N, Jr., Rusch R, Gratton E, Savini I, Finazzi-Agro A. Denaturation of human Cu/Zn superoxide dismutase by guanidine hydrochloride: a dynamic fluorescence study. *Biochemistry* 1992;31:7224–7230.
178. Furukawa Y, O'Halloran TV. Amyotrophic lateral sclerosis mutations have the greatest destabilizing effect on the apo- and reduced form of SOD1, leading to unfolding and oxidative aggregation. *J Biol Chem* 2005;280:17266–17274.
179. Stathopoulos PB, Rumfeldt JA, Karbassi F, Siddall CA, Lepock JR, Meiering EM. Calorimetric analysis of thermodynamic stability and aggregation for apo and holo amyotrophic lateral sclerosis-associated Gly-93 mutants of superoxide dismutase. *J Biol Chem* 2006;281:6184–6193.
180. Lindberg MJ, Tibell L, Oliveberg M. Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic

- lateral sclerosis: decreased stability of the apo state. *Proc Natl Acad Sci USA* 2002;99:16607–16612.
181. Lynch SM, Boswell SA, Colon W. Kinetic stability of Cu/Zn superoxide dismutase is dependent on its metal ligands: implications for ALS. *Biochemistry* 2004;43:16525–16531.
 182. Lynch SM, Colon W. Dominant role of copper in the kinetic stability of Cu/Zn superoxide dismutase. *Biochem Biophys Res Commun* 2006;340:457–461.
 183. Kayatekin C, Zitzewitz JA, Matthews CR. Zinc binding modulates the entire folding free energy surface of human Cu,Zn superoxide dismutase. *J Mol Biol* 2008;384:540–555.
 184. Pantoliano M, McDonnell PJ, Valentine JS. Reversible loss of metal ions from the zinc binding site of copper-zinc superoxide dismutase: the low pH transition. *J Am Chem Soc* 1979;101:6454–6456.
 185. Pantoliano M, Valentine JS, Mamone RJ, Scholler DM. pH Dependence of metal ion binding to the native zinc site of bovine erythrocuprein (superoxide dismutase). *J Am Chem Soc* 1982;104:1717–1723.
 186. Danielsson J, Kurnik M, Lang L, Oliveberg M. Cutting off functional loops from homodimeric enzyme superoxide dismutase 1 (SOD1) leaves monomeric beta-barrels. *J Biol Chem* 2011;286:33070–33083.
 187. Potter SZ, Zhu H, Shaw BF, Rodriguez JA, Doucette PA, Sohn SH, Durazo A, Faull KF, Gralla EB, Nersessian AM, Valentine JS. Binding of a single zinc ion to one subunit of copper-zinc superoxide dismutase apoprotein substantially influences the structure and stability of the entire homodimeric protein. *J Am Chem Soc* 2007;129:4575–4583.
 188. Svensson A-KE, Bilsel O, Kayatekin C, Adefusika JA, Zitzewitz JA, Matthews CR. Metal-free ALS variants of dimeric human Cu,Zn-superoxide dismutase have enhanced populations of monomeric species. *PLoS One* 2010;5:e10064.
 189. Nakano R, Inuzuka T, Kikugawa K, Takahashi H, Sakimura K, Fujii J, Taniguchi N, Tsuji S. Instability of mutant Cu/Zn superoxide dismutase (Ala4Thr) associated with familial amyotrophic lateral sclerosis. *Neurosci Lett* 1996;211:129–131.
 190. Watanabe Y, Kono Y, Nanba E, Ohama E, Nakashima K. Instability of expressed Cu/Zn superoxide dismutase with 2 bp deletion found in familial amyotrophic lateral sclerosis. *FEBS Lett* 1997;400:108–112.
 191. Winterbourn CC, Domigan NM, Broom JK. Decreased thermal stability of red blood cell glu100→gly superoxide dismutase from a family with amyotrophic lateral sclerosis. *FEBS Lett* 1995;368:449–451.
 192. Cardoso RM, Thayer MM, DiDonato M, Lo TP, Bruns CK, Getzoff ED, Tainer JA. Insights into Lou Gehrig's disease from the structure and instability of the A4V mutant of human Cu,Zn superoxide dismutase. *J Mol Biol* 2002;324:247–256.
 193. Shaw BF, Valentine JS. How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein? *Trends Biochem Sci* 2007;32:78–85.
 194. Lyons TJ, Nersessian A, Huang H, Yeom H, Nishida CR, Graden JA, Gralla EB, Valentine JS. The metal binding properties of the zinc site of yeast copper-zinc superoxide dismutase: implications for amyotrophic lateral sclerosis. *J Biol Inorg Chem* 2000;5:189–203.
 195. Nordlund A, Leinartaite L, Saraboji K, Aisenbrey C, Grobner G, Zetterstrom P, Danielsson J, Logan DT, Oliveberg M. Functional features cause misfolding of the ALS-provoking enzyme SOD1. *Proc Natl Acad Sci USA* 2009;106:9667–9672.
 196. Kayatekin C, Zitzewitz JA, Matthews CR. Disulfide-reduced ALS variants of Cu, Zn superoxide dismutase exhibit increased populations of unfolded species. *J Mol Biol* 2010;398:320–331.
 197. Tiwari A, Hayward LJ. Familial amyotrophic lateral sclerosis mutants of copper/zinc superoxide dismutase are susceptible to disulfide reduction. *J Biol Chem* 2003;278:5984–5992.
 198. Hough MA, Grossmann JG, Antonyuk SV, Strange RW, Doucette PA, Rodriguez JA, Whitson LJ, Hart PJ, Hayward LJ, Valentine JS, Hasnain SS. Dimer destabilization in superoxide dismutase may result in disease-causing properties: structures of motor neuron disease mutants. *Proc Natl Acad Sci USA* 2004;101:5976–5981.
 199. Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, Getzoff ED, Hu P, Herzfeldt B, Roos RP, Warner C, Deng G, Soriano E, Smyth C, Parge HE, Ahmed A, Roses AD, Hallewell RA, Pericak-Vance MA, Siddique T. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* 1993;261:1047–1051.
 200. Antonyuk S, Elam JS, Hough MA, Strange RW, Doucette PA, Rodriguez JA, Hayward LJ, Valentine JS, Hart PJ, Hasnain SS. Structural consequences of the familial amyotrophic lateral sclerosis SOD1 mutant His46Arg. *Protein Sci* 2005;14:1201–1213.
 201. Tiwari A, Hayward LJ. Mutant SOD1 instability: implications for toxicity in amyotrophic lateral sclerosis. *Neurodegener Dis* 2005;2:115–127.
 202. Rodriguez JA, Valentine JS, Eggers DK, Roe JA, Tiwari A, Brown RH, Jr., Hayward LJ. Familial amyotrophic lateral sclerosis-associated mutations decrease the thermal stability of distinctly metalated species of human copper/zinc superoxide dismutase. *J Biol Chem* 2002;277:15932–15937.
 203. Rodriguez JA, Shaw BF, Durazo A, Sohn SH, Doucette PA, Nersessian AM, Faull KF, Eggers DK, Tiwari A, Hayward LJ, Valentine JS. Destabilization of apoprotein is insufficient to explain Cu,Zn-superoxide dismutase-linked ALS pathogenesis. *Proc Natl Acad Sci USA* 2005;102:10516–10521.
 204. Vassall KA, Stubbs HR, Primmer HA, Tong MS, Sullivan SM, Sobering R, Srinivasan S, Briere LA, Dunn SD, Colon W, Meiering EM. Decreased stability and increased formation of soluble aggregates by immature superoxide dismutase do not account for disease severity in ALS. *Proc Natl Acad Sci USA* 2011;108:2210–2215.
 205. Pardo CA, Xu Z, Borchelt DR, Price DL, Sisodia SS, Cleveland DW. Superoxide dismutase is an abundant component in cell bodies, dendrites, and axons of motor neurons and in a subset of other neurons. *Proc Natl Acad Sci USA* 1995;92:954–958.
 206. Rakshit R, Robertson J, Vande Velde C, Horne P, Ruth DM, Griffin J, Cleveland DW, Cashman NR, Chakrabarty A. An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS. *Nat Med* 2007;13:754–759.
 207. Bosco DA, LaVoie MJ, Petsko GA, Ringe D. Proteostasis and movement disorders: Parkinson's disease and amyotrophic lateral sclerosis. *Cold Spring Harbor Perspect Biol* 2011;3:a007500.
 208. Bendotti C, Marino M, Cheroni C, Fontana E, Crippa V, Poletti A, De Biasi S. Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. *Progr Neurobiol* 2012;97:101–126.
 209. Kabashi E, Durham HD. Failure of protein quality control in amyotrophic lateral sclerosis. *Biochim Biophys Acta* 2006;1762:1038–1050.
 210. Hwang YM, Stathopoulos PB, Dimmick K, Yang H, Badiee HR, Tong MS, Rumfeldt JA, Chen P, Karanassios V, Meiering EM. Non-amyloid aggregates arising from mature Cu/Zn superoxide dismutases resemble those observed in amyotrophic lateral sclerosis. *J Biol Chem* 2010;285:41701–41711.
 211. Rakshit R, Crow JP, Lepock JR, Kondejewski LH, Cashman NR, Chakrabarty A. Monomeric Cu,Zn-superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and familial amyotrophic lateral sclerosis. *J Biol Chem* 2004;279:15499–15504.
 212. Rakshit R, Cunningham P, Furtos-Matei A, Dahan S, Qi XF, Crow JP, Cashman NR, Kondejewski LH, Chakrabarty A. Oxidation-induced misfolding and aggregation of superoxide dismutase and its implications for amyotrophic lateral sclerosis. *J Biol Chem* 2002;277:47551–47556.

213. Andrus PK, Fleck TJ, Gurney ME, Hall ED. Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 1998;71:2041–2048.
214. Beal MF, Ferrante RJ, Browne SE, Matthews RT, Kowall NW, Brown RH, Jr. Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann Neurol* 1997;42:644–654.
215. Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, Kowall NW, Brown RH, Jr., Beal MF. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem* 1997;69:2064–2074.
216. Barber SC, Shaw PJ. Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. *Free Radic Biol Med* 2010;48:629–641.
217. Poon HF, Hensley K, Thongboonkerd V, Merchant ML, Lynn BC, Pierce WM, Klein JB, Calabrese V, Butterfield DA. Redox proteomics analysis of oxidatively modified proteins in G93A-SOD1 transgenic mice—a model of familial amyotrophic lateral sclerosis. *Free Radic Biol Med* 2005;39:453–462.
218. Shaw PJ, Eggett CJ. Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. *J Neurol* 2000;247(Suppl 1):I17–I27.
219. Simpson EP, Yen AA, Appel SH. Oxidative stress: a common denominator in the pathogenesis of amyotrophic lateral sclerosis. *Curr Opin Rheumatol* 2003;15:730–736.
220. Mulligan VK, Kerman A, Laister RC, Sharda PR, Arslan PE, Chakrabarty A. Early steps in oxidation-induced SOD1 misfolding: implications for non-amyloid protein aggregation in familial ALS. *J Mol Biol* 2012;421:631–652.
221. Baures PW, Oza VB, Peterson SA, Kelly JW. Synthesis and evaluation of inhibitors of transthyretin amyloid formation based on the non-steroidal anti-inflammatory drug, flufenamic acid. *Bioorg Med Chem* 1999;7:1339–1347.
222. Baures PW, Peterson SA, Kelly JW. Discovering transthyretin amyloid fibril inhibitors by limited screening. *Bioorg Med Chem* 1998;6:1389–1401.
223. Miroy GJ, Lai Z, Lashuel HA, Peterson SA, Strang C, Kelly JW. Inhibiting transthyretin amyloid fibril formation via protein stabilization. *Proc Natl Acad Sci USA* 1996;93:15051–15056.
224. Oza VB, Petrassi HM, Purkey HE, Kelly JW. Synthesis and evaluation of anthranilic acid-based transthyretin amyloid fibril inhibitors. *Bioorg Med Chem Lett* 1999;9:1–6.
225. Ray SS, Nowak RJ, Brown RH, Jr., Lansbury PT, Jr. Small-molecule-mediated stabilization of familial amyotrophic lateral sclerosis-linked superoxide dismutase mutants against unfolding and aggregation. *Proc Natl Acad Sci USA* 2005;102:3639–3644.
226. Bugyei-Twum A. Inhibition of transthyretin fibrillogenesis using a conformation specific antibody. M.Sc. Thesis. Toronto, ON: University of Toronto; 2012. pp 68.
227. Quintas A, Saraiva MJ, Brito RM. The amyloidogenic potential of transthyretin variants correlates with their tendency to aggregate in solution. *FEBS Lett* 1997;418:297–300.
228. Quintas A, Saraiva MJ, Brito RM. The tetrameric protein transthyretin dissociates to a non-native monomer in solution. A novel model for amyloidogenesis. *J Biol Chem* 1999;274:32943–32949.
229. Quintas A, Vaz DC, Cardoso I, Saraiva MJ, Brito RM. Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. *J Biol Chem* 2001;276:27207–27213.
230. Lindberg MJ, Bystrom R, Boknas N, Andersen PM, Oliveberg M. Systematically perturbed folding patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. *Proc Natl Acad Sci USA* 2005;102:9754–9759.
231. Lindberg MJ, Normark J, Holmgren A, Oliveberg M. Folding of human superoxide dismutase: disulfide reduction prevents dimerization and produces marginally stable monomers. *Proc Natl Acad Sci USA* 2004;101:15893–15898.
232. Nordlund A, Oliveberg M. Folding of Cu/Zn superoxide dismutase suggests structural hotspots for gain of neurotoxic function in ALS: parallels to precursors in amyloid disease. *Proc Natl Acad Sci USA* 2006;103:10218–10223.
233. Svensson A-KE, Bilsel O, Kondrashkina E, Zitzewitz JA, Matthews CR. Mapping the folding free energy surface for metal-free human Cu,Zn superoxide dismutase. *J Mol Biol* 2006;364:1084–1102.
234. Vassall KA, Stathopoulos PB, Rumfeldt JA, Lepock JR, Meiering EM. Equilibrium thermodynamic analysis of amyotrophic lateral sclerosis-associated mutant apo Cu,Zn superoxide dismutases. *Biochemistry* 2006;45:7366–7379.
235. Silva N, Jr., Grattan E, Mei G, Rosato N, Rusch R, Finazzi-Agro A. Molten globule monomers in human superoxide dismutase. *Bioophys Chem* 1993;48:171–182.
236. Khare SD, Caplow M, Dokholyan NV. The rate and equilibrium constants for a multistep reaction sequence for the aggregation of superoxide dismutase in amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 2004;101:15094–15099.
237. Rumfeldt JA, Lepock JR, Meiering EM. Unfolding and folding kinetics of amyotrophic lateral sclerosis-associated mutant Cu,Zn superoxide dismutases. *J Mol Biol* 2009;385:278–298.
238. Ip P, Mulligan VK, Chakrabarty A. ALS-causing SOD1 mutations promote production of copper-deficient misfolded species. *J Mol Biol* 2011;409:839–852.
239. Banci L, Bertini I, Boca M, Calderone V, Cantini F, Girotto S, Vieru M. Structural and dynamic aspects related to oligomerization of apo SOD1 and its mutants. *Proc Natl Acad Sci USA* 2009;106:6980–6985.
240. Ding F, Furukawa Y, Nukina N, Dokholyan NV. Local unfolding of Cu, Zn superoxide dismutase monomer determines the morphology of fibrillar aggregates. *J Mol Biol* 2012;421:548–560.
241. Hu J, Chen K, Ni B, Li L, Chen G, Shi S. A novel SOD1 mutation in amyotrophic lateral sclerosis with a distinct clinical phenotype. *Amyotroph Lateral Scler* 2012;13:149–154.