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Chapter 9

PRION-LIKE MECHANISMS IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Prion diseases, such as Creutzfeldt–Jakob disease, are caused by a unique class of proteinaceous infectious agents. Pathologically misfolded prion protein can catalyze the conversion of its natively folded counterpart into a misfolded and toxic form, which serves as a recruiting seed for further rounds of protein misfolding that can spread intercellularly through the nervous system. Amyotrophic lateral sclerosis (ALS), a lethal disease characterized by progressive degeneration of motor neurons is currently understood as a classic proteinopathy, although its etiology remains unclear. The disease is typified by the formation of inclusions consisting of misfolded and/or aggregated protein within motor neurons that contribute to neurotoxicity. Recent work has identified prion-like properties in proteins involved in ALS pathology, including their participation in intracellular recruitment, cell-to-cell transmission and even interorganismal transmission. Along with the transmission of naked protein aggregates, experimental evidence suggests that intercellular spread may be facilitated through the ongoing release and uptake of small membranous extracellular vesicles, containing or decorated with disease-specific misfolded proteins, a mechanism already observed for classical prion diseases. Prion-like propagation and transmission may provide the molecular basis for the clinically observed contiguous spread of the disease through the neuroaxis. The inclusion of ALS as a prion-like disease is a relatively recent development, but the evidence and observations supporting it have come at a furious pace. The goal of this chapter is to summarize the current state of knowledge of prion-like proteins in ALS and their mechanisms of transmission between cells and organisms.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive, but ultimately fatal neuromuscular disease characterized by systematic degeneration of the upper and lower motor neurons resulting in weakening and subsequent atrophy of muscles of the limbs, as well as those involved in speech and swallowing [1, 2]. Interestingly, cognitive function is usually spared with only 5% of patients developing frank frontotemporal dementia (FTD) [3]. Clinical onset usually occurs in individuals over 40 and is the most common form of motor neuron disease world-wide with a global incidence of about two in 100,000. Half of affected individuals die within 3 years, and less than 20% survive for more than 5 years [4]. Clinical progression of ALS is relentless and spreads by one of two observable patterns: single focal point initiation, where symptoms appear to begin at a single site and subsequently spreads through adjacent neuroanatomical regions in a spatiotemporal manner [5], or *via* multifocal initiation, where several regions are initially affected, but each focal point serves as the epicenter for contiguous spread of pathology through local propagation [6]. These patterns of disease initiation are highly reminiscent of the spread of prion disease and thus could be explained through the prion-like mechanism of propagated protein misfolding. In addition, prion-like protein misfolding could account for how a mutant or even wild-type protein can dominate pathogenesis of a phenotypically diverse disease such as seen for ALS, akin to when the normal cellular isoform of prion protein (PrP^{C}) is converted to its pathologically dominant form, PrP^{Sc} in prion disease.

The etiology of ALS remains unknown and the mechanisms leading to the selective vulnerability and degeneration of motor neurons in ALS are not well understood; however similar to other neurodegenerative diseases such as Alzheimer disease and Creutzfeldt–Jakob disease (CJD), the disease can be divided into two categories, sporadic and familial (inheritable). 90-95% of ALS cases are sporadic (SALS) where only some predisposing gene mutations have been identified, such as the ataxin-2 intermediate repeat expansions [7]. The remainder of cases are familial (FALS) [8], which are predominantly associated with Mendelian-inherited, primarily autosomal dominant mutations in genes encoding Cu/Zn superoxide dismutase (SOD1), TAR-DNA binding protein 43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), and C9ORF72, but have been associated with mutations in other genes as well (reviewed in [9]) [10, 11, 12, 13]. ALS-associated mutations involving several of these genes increase the propensity of the respective protein to misfold, aggregate and form fibrils [14]. Although the majority of ALS cases are sporadic with no identifiable mutations, pathological forms of these proteins are often present within proteinaceous inclusions found in a large subset of ALS patients, strongly suggesting that protein misfolding plays a key role in disease pathogenesis and/or pathology [15, 16]. In addition, both categories of disease show very similar clinical presentations, suggesting that a common downstream pathogenic mechanism, regardless of disease origin, may lie at the heart of the disease [17].

The post-mortem pathology of ALS patients typically features loss of motor neurons in the brain stem and ventral horn of the spinal cord, accompanied with signs of inflammation such as astrocyte activation and proliferation of microglia [18]. Spinal cord histology often reveals abnormal accumulations of ubiquitinated proteinaceous inclusions in motor neurons and neural accessory cells, which are thought to be the result of aggregated misfolded protein.

Protein misfolding can be triggered by a multitude of factors, including genetic mutation, aberrant oxidation and post-translational modification, dysfunctional proteostasis and seeded polymerization [19]. For example, in FALS cases where a SOD1 mutation is identified, the primary component of these protein inclusions is SOD1 itself [20]. Curiously, in sporadic cases in which SOD1 mutations have been excluded, there is also evidence supporting the presence of misfolded SOD1 [21, 22, 23, 24], suggesting a more expanded role for SOD1 in ALS pathology, although this observation is not one of consensus [25]. A more universally agreed observation is that ubiquitinated and phosphorylated TDP-43 is a primary component of cytosolic inclusions in the vast majority of SALS, in combination with its depletion from the nucleus, where the majority of native TDP-43 normally resides [26]. The presence of misfolded/aggregated protein in ALS, along with the distinct spatiotemporal spread of pathology though the spinal cord makes it a likely candidate as a disease with prion-like mechanistic properties. This chapter will examine the current state of knowledge regarding the prion-like properties of proteins associated with ALS pathology (summarized in Table 1) as well as their possible mechanisms of transmission within and between cells, as well as transmission between organisms.

Table 1. Summary of known evidence of prion-like phenomena in ALS-associated proteins

Protein	Seeded aggregation/ induction of misfolding *in vitro*	Seeded aggregation in cell culture	Seeded aggregation *in vivo*	Intercellular transmission in cell culture	Interorganismal transmission
SOD1	Yes [27, 28].	Yes [28, 29].	Indirect evidence only [30,31].	Yes [24,29].	Yes, under specific experimental conditions [32].
TDP-43	Yes [144].	Yes [33, 34].	n.d.	Yes [34].	n.d.
FUS	Yes [35].	n.d.	n.d.	n.d.	n.d.
C90RF72	Only self-aggregation of DPRs [36, 37].	n.d.	n.d.	n.d.	n.d.
EWRS1	Only self-aggregation *via* prion-like domain [38].	n.d.	n.d.	n.d.	n.d.
TAT15	Only self-aggregation *via* prion-like domain [38].	n.d.	n.d.	n.d.	n.d.
hnRNPA2B1/A1	Yes [39]	Accelerated recruitment into stress granules [39]	n.d.	n.d.	n.d.

n.d., not determined.

THE ROLE OF SOD1 IN ALS PATHOLOGY

In 1993, Rosen et al., identified the first protein to be implicated in familial ALS: Cu/Zn superoxide dismutase (SOD1), a highly abundant and ubiquitously-expressed cytosolic protein whose function is primarily to serve as a free-radical defense enzyme that converts highly toxic free-radical superoxide produced within the cell to less harmful molecules hydrogen peroxide and water [40, 41]. SOD1 mutations occur in 20% of familial ALS patients and have also been detected in a small subset of sporadic ALS patients [42]. To date, over 180 disease-causing mutations have been identified in the 154 amino-acid long protein [10]. Mutant SOD1 proteins display a number of thermodynamic signatures conducive to misfolding, including decreased thermal stability, increased susceptibility to mechanical deformation, increased propensity to monomerize, decreased affinity for metals, and accelerated unfolding under stress conditions [43, 44]. This can lead to the formation of a pool of misfolded SOD1 protein in the cell, potentially through the depletion of copper or structurally-stabilizing zinc under stress [43, 45]. Ultimately, SOD1 aggregation occurs through a mechanism by which the highly-stable native SOD1 homodimer is disrupted producing misfolded monomer intermediates that can be incorporated into higher-order structures [46, 47, 48]. Genetic mutation is not the only way to destabilize, misfold and aggregate SOD1. Aberrant oxidation or post-translational modification of wild-type (WT) SOD1 has been observed to mimic the aggregation-prone effects of mutant SOD1 *in vitro* [47, 49, 50] in a concentration-dependent manner [47]. There is increasing evidence that all types of ALS, including non-SOD1-linked familial and sporadic cases are associated with SOD1 misfolding, oxidation and subsequent aggregation [51]. Inclusions containing aggregated SOD1 have been detected in spinal cord tissues from both FALS and SALS patients [52, 53, 54] in addition to biochemical, genetic and immunological evidence of misfolded SOD1 in cases of mutant SOD1-excluded SALS [21, 22, 23, 24, 55, 56]. Misfolded SOD1 is therefore a prime candidate as a common molecular determinant for all forms of ALS and may play a key role in disease pathogenesis.

The mechanisms by which misfolded SOD1 causes or contributes to ALS pathology are not yet fully understood. Mutations in SOD1 often impair enzymatic function; however loss of superoxide dismutase function is not the primary cause of disease as *SOD1* knockout mice do not develop an ALS-like motor neuron disease [57], but rather a distinctly different motor axonopathy [58]. Conversely, a mutant *SOD1* transgenic mouse developed a progressive motor neuron disease despite increased SOD1 enzymatic activity over and above the mouse endogenous protein [59]. Instead, aberrant folding and subsequent aggregation of SOD1 conferred by mutation, oxidation or other cell stresses is generally thought to acquire a toxic gain of function [57, 59, 60], although the precise nature of this toxicity and its specificity for motor neurons remain to be elucidated. Experimentally, all transgenic mouse strains expressing ALS-causing SOD1 mutants develop cytosolic inclusions in motor neurons and accessory neural cells that coincide with the onset of motor neuron disease and increase in size and number throughout the course of disease progression [61, 62]. When misfolded, SOD1 can react non-specifically with a variety of substrates, causing it to actually become a net producer of reactive oxygen and nitrogen species [63], as opposed to performing its native function as a free-radical scavenger. Aberrant production of free radicals can lead to damage of cellular protein, lipid, and nucleic acid [64, 65]. Toxicity of misfolded SOD1 has also been

attributed to other deleterious cellular effects including: cytoskeletal and mitochondrial disruption, caspase activation, microglial activation, and proteasomal and autophagy pathway disruption [66, 67, 68]. With regard to the latter pathways, pathological SOD1 appears to exert its effects on the protein homeostatic pathways in ALS models even before disease symptoms appear, suggesting other disease functions outside of the inefficient clearance of free radicals within the cell. For example, pathways of the unfolded protein response (UPR) are activated in G93A-SOD1 transgenic mice before the earliest signs of disease are observed [69]. In addition, misfolded mutant SOD1 has been observed to induce dysfunction in both autophagy and the proteasome-dependent degradation pathways [70, 71]. Coincidently, reduced proteasome subunit levels and activity are a common molecular signature in both FALS and SALS patients [72], suggesting deficits in proteasome activity are part of the general molecular disease pathology in humans and may be linked to SOD1 misfolding/aggregation.

PRION-LIKE CONVERSION OF SOD1

The central dogma of the prion hypothesis, as outlined by Prusiner in 1982 [73], stipulates that protein alone is sufficient to act as an infectious agent. On a molecular level, a prion-like protein is often defined as a pathologically misfolded protein that can impart its misfold onto a neighbouring native protein through physical interaction, thus providing more template for subsequent rounds of conversion. Although the breadth of experimental evidence is not as well-established compared to the prion protein itself, an ever-growing body of evidence in the literature suggests that misfolded SOD1 has the ability to transfer conformational information from one SOD1 molecule to another, meeting at least the intermolecular requirements of prion-like activity [27, 28, 29].

Native functional SOD1 enzyme exists as a soluble ubiquitously-expressed homodimer that makes up 1-2% of the total protein of neurons and other neural accessory cells [74]. Monomers are bound to one another *via* hydrophobic interaction at the dimer interface. Furthermore, each monomer contains an intramolecular disulfide bond, believed to contribute to the high conformational stability and resistance to proteolytic digestion of native dimeric SOD1 [75]. Despite its intrinsic stability, destabilization of the enzyme is apparent when the protein monomers are mutated and/or aberrantly oxidized, making them highly susceptible to misfold and form multimeric species and aggregates. More than 180 *SOD1* mutations have been identified in ALS patients [10] affecting at least 75 of the 154 amino acids of the SOD1 protein [76]. Aggregation of mutant misfolded SOD1 inside the cell may not simply be a random process as ubiquitinated misfolded SOD1 is found to cluster into distinct aggregates *via* the juxtanuclear quality control (JUNQ) pathway as a means of sequestering potentially toxic protein from the rest of the cellular machinery [77]. Under denaturing conditions wild-type and mutant forms of SOD1 can spontaneously form aggregates and fibrils *in vitro* [27], where the relative propensity for aggregation is dependent upon the SOD1 mutant variant [78]. *In vivo* SOD1 fibril formation has been observed in transgenic mice expressing mutant SOD1 [79, 80]; however, the formation of amyloid fibrils does not appear to be a consistent feature of SOD1 in human disease as SOD1-positive inclusions from FALS patients do not stain with amyloid-specific dyes [25].

Observations from *in vivo* studies have provided the first, albeit indirect observations of intramolecular conversion of native SOD1 to a pathological form. Acceleration of motor neuron degeneration in mutant SOD1 mouse models is observed upon co-expression of human WTSOD1 [31, 81], possibly through intermolecular stabilization of mutant species *via* WTSOD1 interactions [82] or *via* formation of non-native disulfide interactions that induce insoluble aggregate formation [83].

In addition, co-aggregation of mutant and WTSOD1 has also been detected in tissue derived from familial ALS patients [30], suggestive of intermolecular conversion occurring in patients. More recently, direct evidence of intermolecular WTSOD1 conversion to a misfolded isoform has been observed in human mesenchymal and neuronal cell lines [23, 28]. Expression of misfolded human SOD1 mutants can convert resident WTSOD1 to a misfolded form that is revealed by conformation-specific antibodies that recognize epitopes only accessible when the protein is in a misfolded disease state. The resultant misfolded WTSOD1 in mutant SOD1-transfected cells is highly protease sensitivity, an indicator of overall structural loosening of the polypeptide backbone. Conversion of WTSOD1 to a misfolded conformer by mutant SOD1 has also been shown to occur in a cell-free system, thus eliminating any extraneous protein, lipid or nucleic acid co-factors from the process [28] and further supporting the 'protein-only' paradigm of prion-like protein misfolding.

Interaction between misfolded SOD1 template and native WTSOD1 substrate is a sequence- and/or structure-dependent process akin to the species barrier observed for classical prion diseases such as chronic wasting disease, a transmissible spongiform encephalopathy that is thus far restricted to certain cervid species [84], as mutant human SOD1 does not convert endogenous WTSOD1 to a misfolded species in mouse cells. WTSOD1 conversion appears dependent upon the presence of a single amino acid residue, tryptophan (Trp) at position 32 [28], indicating a potential point of contact between the converting and the converted species that is separate from the dimer interface region, once thought as a more intuitive point of contact between SOD1 molecules. Trp32 is the only tryptophan residue present in the human WTSOD1 amino acid sequence and has previously been identified as a site prone to oxidative modification and a potentiator of aggregation [85]. For example, oxidation of Trp32 caused by SOD1 bicarbonate-dependent peroxidase activity has been shown to trigger SOD1 aggregation, including covalently cross-linking of monomers through ditryptophan bonds [86].

Substitution of the tryptophan by a serine (present in the mouse SOD1 homolog) in ALS-associated mutants of SOD1 serving as 'seed' for the conversion of WTSOD1 fails to induce WTSOD1 misfolding in human cell lines [28], likely explaining why mutant human SOD1 is inert in converting WTSOD1 in mouse cells. The structure/sequence requirements for intermolecular conversion of SOD1 is also seen *in vivo* as human WTSOD1 does not accelerate motor neuron disease in mice expressing mutant murine SOD1 with a G86R substitution [87], analogous to the human G85R mutation linked to FALS. Furthermore, murine SOD1 is not incorporated into aggregates of human mutant SOD1 [31, 83, 88].

PROPAGATION OF SOD1 MISFOLDING

As presented in the previous section, there is ample evidence supporting the intermolecular conversion of WTSOD1 by mutant SOD1; however the mechanism by which this process occurs remains unclear. For the prion protein, two possible mechanisms of propagation have been proposed: nucleated polymerization and template directed misfolding [89]. Nucleated polymerization occurs when the misfolded protein species is intrinsically less stable as a monomer but becomes more stable than its native counterpart when recruited to a multimeric aggregate. When misfolded monomer encounters a nucleating oligomer, the former is incorporated into the latter and eventually leads to fibril elongation, which may break-up into multiple oligomeric seeds that subsequently become the new recruiters. In template-directed misfolding, the misfolded conformer is more stable than the native form but is kinetically inaccessible without catalysis by an interaction with other misfolded conformers; a misfolded SOD1 oligomer becomes a physical template for the misfolding of the native WTSOD1. Once converted, misfolded SOD1 can further propagate by catalyzing additional rounds of conversion, thus forming aggregates and fibril structures in increasing size and number. Evidence for both propagated protein misfolding mechanisms have been observed for SOD1 and suggests that prion-like propagation of both mutant and WTSOD1 likely spans a continuum between the two models. Aggregation of partially unfolded mutant SOD1 may self-propagate primarily by nucleated polymerization. For example, exogenous soluble mutant SOD1 was observed to incorporate into endogenous mutant SOD1 aggregates inside transfected mouse neuroblastoma cells [29]. On the other hand, conversion of WTSOD1 may require structural loosening induced by contact with a mutant or misfolded wild-type template [47] before recruitment to the misfolded SOD1 seed. In support of this notion is the finding that WTSOD1 can only participate in nucleated polymerization upon exposure to low pH and the chaotrope guanidine *in vitro* [21, 27], conditions that destabilize the native state and favour monomer recruitment into aggregates.

The dynamics of mutant SOD1 propagated misfolding depend on the intrinsic aggregation properties and structural stabilities of the different mutant isoforms, and play an essential role in disease progression and patient survival with SOD1-FALS [90]. Crystal structures from recombinant metal-deficient mutant SOD1 proteins reveal assemblies of aligned beta-sheets forming amyloid-like filaments and water-filled nanotubes that result in enzymatic dysfunction [91] suggesting this conformational rearrangement in the metal-deficient enzyme could contribute to SOD1-linked FALS pathology. Recombinant apo-SOD1 without disulphide bonds, ALS mutant SOD1 protein or insoluble SOD1-containing aggregates isolated from mutant SOD1-expressing transgenic mice have all been shown to possess spontaneous fibrillization and seeding activity *in vitro* under various non-physiological conditions [27, 80]. Recent observations have also shown that protein to protein conversion induced by both mutant [29] and over-expressed WTSOD1 [28] can be self-sustaining. Induced misfolding of WTSOD1 can persist even in the absence of the original mutant SOD1 seed in cell culture, suggesting that the induced misfolded SOD1 can act as template for subsequent cycles of misfolding [24]. Similarly, exogenous fibrils of recombinant mutant human SOD1 can be taken up by mouse neuroblastoma cells expressing the same human SOD1 mutant and trigger intracellular aggregation [92].

In the case of propagating WTSOD1 misfolding, it is unclear what the conformational state of the substrate is in prior to conversion. It would be highly thermodynamically unfavorable for natively-folded WTSOD1, or even an enzymatically functional mutant SOD1 in a near-native state to be susceptible to induced misfolding. In fact, co-expression of WTSOD1 is observed to stabilize mutant misfolded SOD1 [93, 94]. FALS-linked mutant SOD1 species are known to have delayed post-translational folding kinetics, thereby increasing the population of partially folded intermediates [95]. Thus, nascent WTSOD1 polypeptide newly-emerged from the cellular translation machinery may provide a thermodynamically favourable substrate for recruitment (Figure 1). However, WTSOD1 does possess relatively quicker folding kinetics compared to mutant isoforms, thus producing significantly smaller populations of partially-folded intermediates. Other mechanisms may be at work in the ALS disease state that slow post-translational folding of WTSOD1, making it more susceptible to induced misfolding; however, such factors have yet to be identified.

INTERCELLULAR TRANSMISSION OF SOD1 MISFOLDING

The regional spread of protein misfolding and aggregate formation is a prevalent feature of neurodegenerative disease, with the spread of misfolded protein seed playing the critical role of expanding the pathology beyond the site of original protein conversion. A misfolded protein that cannot escape the local cellular environment in which it was formed cannot effectively spread pathology to adjacent anatomical regions. The mechanisms of intercellular transmsision of misfolded protein in ALS have yet to be fully elucidated, but various pieces of experimental evidence have provided some insight thay may explain the spatiotemporal spread of disease pathology. Simple release and uptake of soluble misfolded protein, aggregates or proto-fibrils have been observed experiementally in cell lines. For example, exogenous mutant [24, 29] and WTSOD1 [24] aggregates have been shown to efficiently penetrate the cell membrane of neuron-like cells in a macropinocytosis-dependent mechanism, and become self-perpetuating in recruiting soluble SOD1 into insoluble aggregates. Aggregates of misfolded SOD1 likely represent a conformationally and thermodynamically stable form [96] that can better survive the relative hostilities of the extracellular environment than lower-order soluble forms, possibly becoming available when dying neurons release their contents to the extracellular environment.

Although the presence of large proteinaceous inclusions containing SOD1 is a common pathological characteristic in cases of SOD1-FALS , there is a question as to whether these protein structures represent the actual transmissible neurotoxic form of pathological SOD1. In other neurodegenerative diseases, there is growing evidence pointing to *soluble* misfolded protein as the primary toxic species as opposed to large protein aggregates, inclusions or fibrils, which appear for the most part to be pathologically inert [97]. In Alzheimer's disease, it is smaller oligomeric forms of amyloid-β that impair synaptic function [98, 99], plasticity and memory [100]. Likewise, there is growing evidence that pre-fibrillar oligomers of α-synuclein are responsible for disease progression in Parkinson disease [101, 102] as opposed to the hallmark Lewy bodies, which may serve as benign 'dumps' of aggregated protein. A similar situation may exist for misfolded SOD1 in ALS. For example, misfolded SOD1 is detectable in mutant SOD1-excluded sporadic and familial cases, but not typically in the form of large protein inclusions usually observed in mutant SOD1-associated FALS [21, 22, 24],

suggesting that misfolded SOD1 in SALS is likely present in the form of smaller soluble structures that are not readily detectable by gross histochemical staining of tissue sections. Mechanistically, the spread of ALS pathology would likely occur prior to neuronal cell death, *via* a more active cellular process.

There is increasing evidence supporting the role of extracellular vesicles (EVs) in the pathobiology of neurodegenerative disease as a means of transmitting toxic proteins from region to region [103]. EVs are membrane-bounded vesices that range in size from 30-1000 nm and are released by various cell types including motor neurons and neural accessory cells. EVs have been shown to carry an assortment of macromolecular cargo inclduing mRNAs, miRNAs, lipds and proteins [104, 105]. Exosomes represent an EV subtype, ranging in size from 30-80 nm that arise from the inward budding of the late endosome and are held within the cell by mutlivesicular bodies (MVB). MVBs are normally involved in the lysosomal pathway, trafficking proteins to the lysosome for proteolysis. Alternatively, MVBs can fuse with the plasma membrane, leading to the release of its intraluminal vesicles into the extracellular milieu as exosomes [106]. Exosomes have been increasingly implicated in the spread of misfolded or aggregated proteins involved in a number of neurodegenerative proteinopathies, such as Alzheimer's disease [107], Parkinson's disease [108, 109] and the prion diseases [110, 111]. It is thought that as misfolded and aggregated proteins accrue in the intracellular environment, it begins to overwhelm the protein clearance pathway leading the cell to release aggregated protein packaged in EVs. Consequently, other cells take up the secreted EVs with their toxic cargo. Evidence continues to build that an analogous EV-associated mechanism may be involved in the pathobiology of ALS (Figure 1). SOD1 itself is a known resident protein of exosomes from multiple cell types and species [112]. Subsequent studies have identified exosomes as a secretion mechanism for both wild-type and mutant SOD1 in a motor neuron-like cell model [113], potentially mediated by chromogranins [114], acidic components of neurotransmitter-enriched secretory vesicles [115] that could serve as a secretory chaperone. However, expression of mutant SOD1 has also been shown to impair its own secretion, possibly contributing to intraneuronal toxicity [116], thus confounding the precise role of exosomes in mutant SOD1 transmission. Other evidence suggests that mutant SOD1 oligomers accumulate in the endoplasmic reticulum (ER) -Golgi compartments of the endocytic pathway prior to its subsequent secretion [117]. In another case, exogenous mutant and misfolded WT SOD1 taken up by neuronal cells inhibits protein transport between the Golgi and ER, ultimately leading to Golgi fragmentation, ER stress and apoptosis [118]. In addition, Golgi fragmentation is observed as an early event in the pathology of a mutant SOD-ALS mouse model [119], further complicating a potential role of the secretory pathway in pathological SOD1 transmission during disease. Other work has established that both mutant and wild-type misfolded SOD1 can be secreted from neuron-like cells *via* exosomes [120]; these exosomes can be subsequently taken up by fresh cells where the misfolded SOD1 cargo provides a template for subsequent induction of protein misfolding [24]. Exosome-independent uptake of SOD1 is not aggregate-specific as aggregated forms are taken up as efficiently as non-aggregated forms. However, the process does show specificity, as an irrelevant cellular aggregate, such as glutathione S-transferase, is not taken up in the same manner as SOD1, suggesting the possible involvement of receptors in this process. Previous work with mouse microglial cells found that scavenger receptors specifically participate in the uptake of aggregated SOD1 [121], whereas heparin sulfate proteoglycans have been shown to be involved in aggregate uptake of tau, alpha-synuclein, and prion protein [122, 123] in other

neurodegenerative diseases. More recently, mouse astrocyte-derived exosomes were observed to efficiently transfer mutant SOD1 to spinal neurons and subsequently cause selective motor neuron death [120], providing further evidence that exosome-mediated transmission of misfolded SOD1 is pathogenic.

INTERORGANISMAL TRANSMISSION OF PATHOLOGICAL SOD1

Misfolded SOD1 appears to satisfy both the intermolecular conversion and intercellular propagation characteristics of prions; however, one could argue that despite fulfilling these biochemical properties, SOD1, and by extension other misfolded proteins involved in neurodegenerative proteinopathies, do not share key traits of the classical prion diseases, namely transmission between organisms [96] and the evidence of strains that vary in their stability, infectivity and toxicity [124]. Moreover, there is little evidence in the literature to date that resolutely confirms the protein-only hypothesis in any of the other neurodegenerative proteinopathies. SOD1 could be considered the lone exception as propagated conversion of WTSOD1 to a misfolded conformer was observed in a completely cell-free environment using purified recombinant protein [28] with the caveat that the solution conditions were only a rough approximation of the intracellular environment. When it comes to organismal infectivity no other misfolded protein comes close to the degree of pathogenicity of PrP^{Sc} in the classical prion diseases. PrP^{Sc} is a highly-stable isoform compared to normal PrP^{C} and represents the epitome of a robust infectious agent that is able to withstand the environmental rigours of animal-to-animal transmission. Conversely, many pathological isoforms of misfolded SOD1 are highly protease-sensitive and unstable [28, 75] making long-term survival outside of an organism or even in the extracellular milieu highly unlikely, although the presence of native wild-type SOD1 has been shown to increase the stability of mutant misfolded SOD1 [125]. The point of entry of prion-like agents is likely another crucial factor for efficient transmission. Interorganismal transmission of propagated SOD1 misfolding would likely need to be directly introduced to the anatomical region of pathology; for ALS this would typically be the spinal cord or brain stem. At the time of writing this chapter only one published account has demonstrated a model for interorganismal transmission of motor neuron disease, which indeed utilizes the aforementioned routes of entry. In this experiment, transgenic mice expressing human mutant G85R-SOD1 fused to a YFP reporter (G85R-SOD1:YFP) at levels too low to induce disease were intracerebroventricularly injected with spinal cord homogenate isolated from diseased G93A-SOD1 mice [32]. Recipient mice showed the induction of G85R-SOD1:YFP aggregation and the progressive development of motor neuron disease, demonstrating for the first time interorganismal transmission of motor neuron disease pathology. Furthermore, the study showed that spinal cord homogenate from G37R-SOD1 transgenic mice does not induce disease, suggesting the possibility of strain-like characteristics for different misfolded isoforms of SOD1 [32]. Superficially, key prion characteristics are observed in this study, however the authors are quick to point out that disease transmission was relatively inconsistent and disease pathology varied considerably from animal to animal, indicating partial penetrance of disease. If misfolded SOD1 pathology is indeed transmissible, its

virulence is several orders of magnitude lower than PrP^{Sc}, the archetypal proteinaceous infectious particle.

TDP-43 MISFOLDING IN DISEASE

The formation of ubiquitin-positive inclusions is a common feature of many neurodegenerative diseases [126]. Two independent discoveries in 2006 identified pathological TAR-DNA binding protein 43 (TDP-43) as a major protein in ubiquitin-positive, tau- and α-synuclein-negative neuronal and glial inclusions, hallmark structures in certain cases of frontotemporal lobar dementia (FTLD), and in the majority of SALS cases [26, 127], providing a common mechanistic link between the two disorders. Interestingly, TDP-43 immunoreactivity has also been observed in inclusions found in Huntington's disease [128], Parkinson's disease [129], and nearly 20% of Alzheimer's disease cases [130], which implicate TDP-43 in a wide spectrum of neurodegenerative disorders. In addition to its translocation from the nucleus to the cytoplasm and subsequent aggregation, pathological TDP-43 also becomes hyperphosphorylated and ubiquitinated [26, 127]. Mutations in the gene encoding TDP-43, *TARDBP*, were subsequently identified in patients with FALS and SALS, suggesting a direct causal nature between the protein and disease [131, 132, 133, 134, 135, 136]. Intriguingly, almost all of the *TARDBP* mutations identified in ALS are located in exon six encoding the C-terminal domain [132, 133] and result in autosomal dominant inheritance [137]. Originally, TDP-43 was thought to be involved in early embryonic development as disruption of *TARDBP* expression is lethal at the embryonic stage [138, 139]. However, the protein is now known to be involved in gene regulation, and mRNA splicing and localization [140]. Studies have determined that TDP-43 mislocalization and aggregation is directly related to disease pathogenesis as opposed to being an end-stage marker of disease. However, the precise role of TDP-43 in disease presently remains ambiguous; it is unclear whether pathology is caused by a toxic gain-of-function due to cytoplasmic mislocalization, loss-of-function due its resultant nuclear depletion, or a combination thereof.

Functionally, TDP-43 is an important regulatory protein in the nervous system: it binds over 6,000 pre-mRNAs, affects the splicing patterns of 965 mRNAs and affects expression levels of another 600 mRNAs [141]. Due to its substantial role in transcription regulation, some propose that the loss of function of pathological TDP-43 in disease can cause neuronal loss due to altered proteostasis of proteins that rely on TDP-43 for their synthesis [142]. Others believe that cytoplasmic mislocalization of pathological TDP-43 is toxic to neurons, potentially by leading to the formation of cytotoxic misfolded SOD1 protein, which could implicate both proteins in a common pathological pathway in ALS [23, 143]. Structurally, the 414 residue TDP-43 protein belongs to the heterogeneous ribonucleoprotein family. In addition to its nuclear localization and export domains that enable it to shuttle sequences of nucleic acid in and out of the nucleus, TDP-43 also contains two RNA recognition motifs (RRM1 and RRM2), and an arginine/glycine-rich C-terminal domain through which it interacts with other proteins [144, 145]. Screening of the entire database of known human proteins using a hidden Markov Model algorithm designed to detect prion-like sequences in yeast found TDP-43 to be the 69^{th} most likely protein to contain a prion-like domain out of 27,879 [146], where the C-terminal region in particular was predicted to be prone to

misfolding [147]. Pathological TDP-43 is abnormally cleaved into the cytotoxic 25- and 35-kDa C-terminal fragments (CTFs) [133], becoming themselves susceptible to hyperphosphorylation, ubiquitination and aggregation [148]. These fragments have been shown to be necessary and sufficient for TDP-43 self-aggregation [140, 149] as even truncation constructs featuring minimal C-terminal portions of the protein are aggregation-competent *in vitro* and in cultured cells [14, 33, 150]. Udan-Johns et al., observed that both endogenous and fluorescent-tagged wild-type human TDP-43 undergo reversible aggregation in the nucleus in response to heat shock [151]. The same study confirms that deletion of the C-terminal prion-like domain markedly reduces aggregation of TDP-43, and that replacement of the prion domain in TDP-43 with a prion domain from TIA-1 recapitulates the heat shock induced aggregation. Yeast models of TDP-43 proteinopathy successfully replicate the cytoplasmic mislocalization and toxicity seen in disease and show that the prion-like domain of TDP-43 is essential for its aggregation and cellular toxicity, demonstrating a proposed role in human disease [149]. Other studies have shown that the prion-like domain is essential for the normal function of the protein as deletion mutants fail to properly splice normal TDP-43 targets while substitution of a yeast prion domain into the C-terminus of TDP-43 successfully rescues function [152].

TDP-43 SHOWS PRION-LIKE PHENOMENA

Evidence is rapidly emerging demonstrating prion-like behavior in TDP-43. It is firmly established that, similar to mutant and/or aberrantly oxidized forms of SOD1, TDP-43 and its derived fragments have a high propensity to aggregate *in vitro* [33, 140] and pathological mutations expressed within the protein can enhance this property [14, 140]. Aggregates of TDP-43 CTFs can form detergent-insoluble fibril-like structures [127, 153] localized to the cytoplasm. Synthetic CTFs were able to also form detergent and heat resistant high molecular weight multimers as well as partially protease-resistant low molecular weight species in primary neurons [14]. Interestingly, the low molecular weight TDP-43 species were able to form neurotoxic β-sheet amyloid fibrils, reminiscent of amyloid-like TDP-43-positive inclusions found in post-mortem ALS spinal cord samples [154], although TDP-43 can also be generally found in non-amyloid forms as well [155]. Investigations into the process by which TDP-43 aggregates and fibrils are formed have revealed prion-like characteristics that contribute to its pathology. TDP-43 can be cross-seeded in cultured cells by poly-Q aggregates , a process dependent upon the protein's glutamine/asparagine (Q/N)-rich prion-like domain [156], resulting in TDP-43 nuclear depletion and accumulation into misfolded aggregates in the cytoplasm. Work by Furukawa et al., has demonstrated that exogenously applied detergent-insoluble aggregates of full-length TDP-43 generated *in vitro* can be taken up by human embryonic kidney cells, and act as seed for the aggregation of endogenous TDP-43 [33]. These induced aggregates are themselves detergent-insoluble and ubiquitinated, similar to that observed for pathological TDP-43 in ALS patients, but are not hyperphosphorylated. Despite this, the results clearly implicate the process of seeded aggregation in TDP-43 pathology. Comparable mechanisms have been implicated in several other notable neurodegenerative proteinopathies, including the prion diseases [157], Alzheimer's disease [158, 159] and Parkinson's disease [160].

Even more convincing self-templating properties have been observed for TDP-43 in a recent study by Nonaka et al., Aggregated TDP-43 isolated from the brains of ALS and frontotemporal lobar degeneration patients exogenously applied to cultured human neuroblastoma cells can serve as seed for the self-propagation of additional ubiquitinated and phosphorylated TDP-43 aggregates [34], with the aggregation of full length TDP-43 preceding the C-terminal cleavage of the protein by caspases [161]. Interestingly, treatment of detergent-insoluble TDP-43 with protease, which degrades the protein to <20 kDa fragments, does not diminish its seeding ability, further suggesting that full-length TDP-43 is not necessary for disease pathogenesis [34]. In addition, Nonaka et al., found that the seeding ability of tissue-derived aggregated TDP-43 is resistant to heat, protease digestion and formic acid, similar to that found for pathological prion protein. Furthermore, TDP-43 aggregates could propagate between cells through several passages, suggesting propagated misfolding may persist even in the absence of the original templating seed, indicative of an authentic prion-like self-propagating mechanism. It was further observed that accumulation of pathological TDP-43 in a self-templating manner leads to proteasomal dysfunction, possibly driving further cytoplasmic accumulation of TDP-43 aggregates in a positive-feedback manner [34,162] thus establishing a solid link between propagated TDP-43 misfolding and disease pathology. The proposed prion-like activities of TDP-43 are summarized in Figure 1.

A key characteristic of infectious prions is the existence of distinct strains, a property that has confounded researchers for decades. Despite the gene encoding PrP having no identifiable genetic mutations, different strains of infectious PrP^{Sc} have been identified possessing stable and distinct incubation periods, patterns of pathology, and disease severity [163].

Although not as firmly established as PrP^{Sc} in the various forms of transmissible spongiform encephalopathies, there is emerging evidence suggesting different strains of TDP-43 may be responsible for distinct pathological subtypes of fronto-temporal lobar degeneration with TDP-43 pathology (FTLD-TDP) based on the type of TDP-43-positive inclusions observed [164]. Furthermore, different structural conformations of sarkosyl insoluble TDP-43 from cortex and spinal cord from ALS and FTLD patients produced distinct phosphorylated TDP-43 fragments *via* protease digestion [165]. The distinct patterns of fragments produced by protease digestion were specific for different TDP-43 proteinopathy subtypes [164], suggesting that different conformational changes to TDP-43 cause different patterns of pathology and disease phenotypes, highly reminiscent of distinct strains of infectious prion protein.

Experimental evidence for the intercellular transmission of pathological TDP-43 [34] could also help to explain the prion-like spatiotemporal spread that is clinically observed in ALS [5]. In support of this, TDP-43 pathology is observed to spread in a four-stage process starting from the agranular frontal neocortex and somatomotor neurons of the spinal cord and brain stem, followed by subsequent spreading to the medial temporal lobe and hippocampus by the last stage [166], a pathological progression highly reminiscent of prion-like spread. Although the mechanism of pathological TDP-43 transmission and propagation has yet to be identified, it has been speculated that pathological TDP-43 could spread transynaptically *via* corticofugal axonal projections using anterograde axonal transport [167]. In addition, exosomes may play a role in the intercellular transmission of pathological TDP-43, similar to that observed for misfolded SOD1, as full-length TDP-43 is enriched in exosome fractions isolated from human neuroblastoma cells treated with seed from ALS patient extract [34],

although other mechanisms of transmissions could also be at play, such as direct uptake of aggregates by the plasma membrane as seen for aggregates of SOD1 [24] (Figure 1).

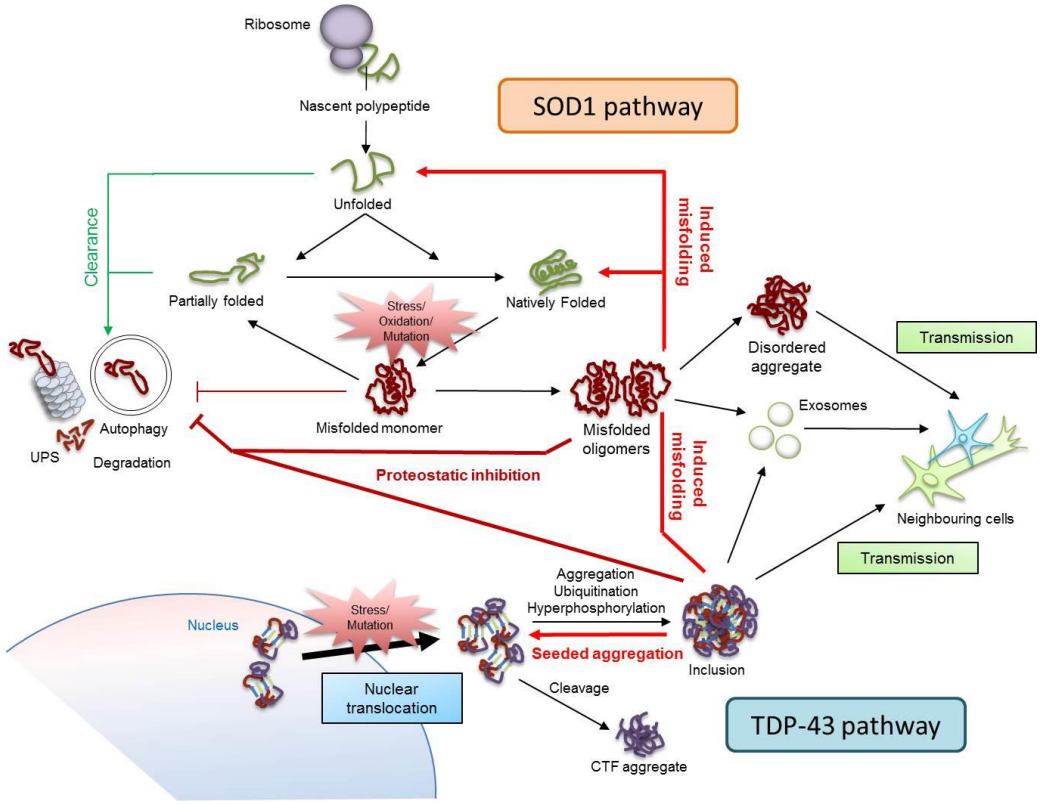

Figure 1. Proposed prion-like mechanisms of misfolded SOD1 and pathological TDP-43 in ALS. Misfolded SOD1, generated through mutation, aberrant oxidation or other cell stressors utilizes post-translational intermediates of WT or mutant SOD1 that remain partially unfolded and susceptible to induced pathological misfolding. Once a misfolded template is present, it can induce subsequent cycles of template-directed misfolding, converting neighbouring native SOD1 molecules into pathological isoforms, which can subsequently form oligomers and aggregates over time. Misfolded SOD1 eventually accumulates in the ER-Golgi system where it can enter the vesicle-mediated secretory pathway, exit the cell *via* exosomes and be taken up by neighbouring cells. Alternatively, large aggregates containing misfolded SOD1 are released and subsequently taken up by neighbouring cells, providing misfolding-competent seed. Misfolded SOD1 itself can act as an inhibitor of both the proteasome and autophagic systems, through as yet identified mechanisms, thereby allowing for aberrantly folded protein to elude proteostasis and build up within the cell. TDP-43 translocation from the nucleus to the cytoplasm can occur *via* mutation or cell stressors. Cytoplasmic TDP-43 has a propensity to aggregate, forming nucleating seeds that recruit other soluble TDP-43 molecules into larger aggregates. Similarly, TDP-43 can be proteolytically cleaved, releasing its CTF, which can form aggregates with other CTFs *via* its prion-like domain. Pathological TDP-43 is known to be released from cells, and has also been detected as a component of exosomes, implicating these vesicles as a mode of transport out of the cell. Evidence also supports a role for pathological TDP-43 in the induction of SOD1 misfolding and dysfunction of the proteostatic machinery.

PRION-LIKE PROPERTIES IN OTHER ALS-ASSOCIATED PROTEINS

Since the initial discovery of TDP-43 involvement in neurodegenerative disease, a host of other RNA-binding proteins, belonging to the heterogeneous ribonucleoprotein family and containing prion-like domains, have been identified and linked to ALS pathobiology. Fused in sarcoma (FUS), a 526 amino-acid RNA-binding transcriptional activator, is structurally and functionally related to TDP-43 [145]. FUS has different binding partners than TDP-43, and the two do not co-localize within pathological inclusions in the cytoplasmic [168, 169] despite their structural, functional and pathological similarities, suggesting the two proteins are involved in pathology *via* distinct, albeit related mechanisms. For example, pathological forms of FUS and TDP-43 have both been observed to induce SOD1 misfolding in cultures cells [23], although the mechanisms for this are currently unknown, in addition to whether or not FUS or TDP-43 acts in a similar fashion in this process.

The C-terminal region of FUS contains a nuclear localization signal (NLS), and glycine-rich domain, while the first 239 amino acids make up a predicted Q/N-rich yeast prion-like domain [147]. Curiously, purified FUS shows a higher propensity to aggregate *in vitro* under native conditions than either SOD1 or TDP-43, requiring only its RNA-binding RRM and glycine-rich RGG domains [169]. The RGG domain of FUS also contains a small prion-like region which opens up the possibility that FUS may self-aggregate using its two prion-like domains [170]. However, FUS mutations that cause ALS are primarily located within the NLS and are believed to primarily contribute to the mislocalization of the protein to the cytoplasm rather than promote misfolding and/or aberrant aggregation [169, 171, 172]. The cytoplasmic accumulation of FUS appears to be enough to induce its aggregation and integration into stress granules (dense foci of RNAs and protein that form in response to certain cell stressors) in cultured neurons [172]. Similar to TDP-43, FUS can be cross-seeded by poly-Q aggregates in cell culture, sequestering the protein into cytoplasmic inclusions in a manner dependent upon its Q/N-rich prion-like domain [156]. Recently, it was shown that the pathogenic FUS mutation, G156E, significantly increases the propensity for both in *vitro* and *in vivo* aggregation of FUS. Spontaneous *in vitro* formation of amyloid-like fibrillar aggregates was observed in mutant but not wild-type FUS, and more importantly, these fibrils served as efficient seeds to trigger the aggregation of wild-type protein [35]. However, at the time of writing this chapter, no experimental evidence has been published demonstrating seeded aggregation of FUS in living cells, although it has been hypothesized that the environment within stress granules, where the concentration of FUS is relatively high may be conducive for the initiation of seeded aggregation, possibly mediated *via* RNAs that could serve as scaffolding molecules [173] and thus not a true prion-like mechanism.

Ewing sarcoma breakpoint region 1 (EWSR1) and TATA-binding protein associated factor 15 (TAF15) are two other proteins that share similar prion-like domain architecture to FUS, are part of the TET family of proteins (reviewed in [174]) and implicated in ALS. TAF15, which serves as a component of the scaffold for assembly of the RNA transcription complex, was identified in a systematic survey used to screen candidates similar to TDP-43 or FUS, which could be involved in ALS [175]. Like other TET family members, TAF15 has a QGSY-rich N-terminal domain which is also predicted to be a prion-like domain [176]. Recent work has demonstrated that TET members bind with each other *via* their N-terminal prion-like domains to form homo- and hetero-complexes, a process which appears to mediate

stress granule formation in normal cells, but can be dysregulated in disease resulting in the cytoplasmic inclusions routinely observed in ALS [38]. EWSR1 is an RNA-binding protein also identified in a screen for proteins with prion-like domains, and disease-causing mutations in EWSR1 were discovered in ALS patients [175]. Further work is needed to determine whether TET family members propagate in a prion-like manner in ALS similar to SOD1 or TDP-43, and whether their prion-like domains play a role in this process. The above studies suggest that RNA-binding proteins normally self-associate through their prion-like domains, and that disease-causing mutations in these domains lead to aggregation, perhaps through unregulated polymerization with each other [38]. However, evidence of self-propagation of protein misfolding or intercellular transmission has yet to be published.

Mutations in genes encoding two other RNA-binding proteins with prion-like domains have been identified in an ALS patient. Pathogenic mutations identified in a case of FALS and an inheritable multisystem proteinopathy have been identified in the prion-like domains of heterogeneous nuclear ribonucleoproteins (hnRNPs) A2B1 and A1 [39]. In their wild-type forms, both proteins show a strong tendency to assemble into self-seeding fibrils, although this process is accelerated in the presence of pathological mutations, incorporating wild-type hnRNP into fibrils in a seeded polymerization-type mechanism [39]. Pathological mutations also promote incorporation of hnRNPA1 and hnRNPA2 into stress granules and the formation of cytoplasmic proteinaceous inclusions in a *Drosphila* disease model, recapitulating the human phenotype, demonstrating a direct relationship between dysregulated polymerization of hnRNP proteins and onset of degenerative disease. It has been noted that diseases associated with cytoplasmic inclusions of proteins containing prion-like domains often show the characteristic spatiotemporal spreading of pathology [176] reminiscent of the clinically observed contiguous spread ALS through the neuroaxis [5]. Taken together, RNA binding proteins with prion-like domains may also transmit propagated protein misfolding *via* self-templating polymerization from cell-to-cell in a manner similar to that observed for SOD1, and to a lesser degree TDP-43, although direct evidence of this has yet to be shown in the literature.

Finally, the recent discovery that expansion of a GGGGCC hexanucleotide repeat within the *C9ORF72* gene is the most common mutation in families with ALS or frontotemporal dementia [177] adds another prominent candidate to the list of ALS-associated proteins with peculiar aggregation dynamics. Hexanucleotide expansion in *C9ORF72* is found in nearly 40% of all FALS cases and even up to 7% in SALS [178]. In normal individuals, the hexanucleotide repeat length is typically no longer than 25-30 units, but in ALS patients possessing a *C9ORF72* mutation, repeat length can exceed 700 [179, 180]. C9ORF72-asscoiated ALS cases typically present with TDP-43 aggregation, and p62-positive and TDP-43-negative ubiquitinated inclusions in the cerebellar and hippocampal regions [181, 182]. Mutation within *C9ORF72* results in the subsequent production of aggregating dipeptide-repeat (DPR) proteins [36, 37], which are generated by an alternative type of translation called repeat-associated non-ATG (RAN) translation [183]. DPRs are the primary components of TDP-43-negative inclusions in ALS patients, although the precise role in pathogenesis remains unknown. There is evidence that supports that DPRs can confer toxicity in cultured cells though mechanisms that disrupt RNA biogenesis [184] and protein trafficking [185]. However, it remains to be seen if C9ORF72 DPRs possess any prion-like characteristics prior to their accumulation into inclusions. Considering the prominence of

C9ORF72 mutations among ALS patients, further characterization into the aggregating behaviour of the DRPs and its relevance to ALS pathology is paramount.

USING THE PRION PARADIGM FOR THERAPY DEVELOPMENT

The prion paradigm of ALS offers new opportunities for therapeutic intervention that go beyond simple neuroprotection currently offered by the sole ALS pharmacological therapeutic, riluzole, available on the market. Increased susceptibility to proteolysis of misfolded ALS proteins, such as SOD1 and TDP-43, may expedite their clearance under conditions of enhanced proteasome activation. A similar strategy has been tried in Huntington's disease in which proteasome activators were overexpressed in Huntington patients' skin fibroblasts or mutant huntingtin-expressing striatal neurons [186]. Conversely, interventions may include stabilization of the native conformer of prion-like proteins through the binding of compounds or other biomolecules. In the case of SOD1, monomerization of the native dimer is known to be an early step in misfolding [47], thus molecules that stabilize the native dimer may impede misfolding. Alternatively, small molecules could be utilized to block self-templating of ALS-associated proteins at the site of protein-protein interaction. Although no compounds have yet to be identified that prohibits interactions between the prion-like domains of TDP-43 or FUS, there is crystallographic evidence of specific small molecules binding at or near the Trp32 residue of SOD1 [187], Trp32 being the residue identified as crucial for misfolded SOD1 self-templating and propagated misfolding [28]. Further study will be required to determine whether these compounds effectively inhibit prion-like activity of misfolded SOD1. Other strategies may focus on the transit of misfolded protein through the extracellular environment.

The work by Grad et al., has established that the intercellular transmission of misfolded SOD1 template can be abrogated immunologically through the use of antibodies directed against epitopes that are only exposed when the protein is aberrantly folded [24], demonstrating that misfolded SOD1 is accessible to immunotherapeutic intervention in the extracellular environment. Furthermore, the association of misfolded SOD1 with exosomes appears inconsequential to antibody binding as it was reported that misfolded SOD1 is immunodetectable on the outside of vesicles [24]; this may or may not be analogous to exosome-associated pathological TDP-43 [34]. Immunotherapeutic strategies targeting misfolded isoforms of SOD1 have been investigated *in vivo* with varying results. Passive immunization *via* intracerebroventricular infusion of G93A-SOD1 transgenic mice with monoclonal antibodies that bind epitopes of misfolded SOD1 that are not present in native WTSOD1 successfully reduced levels of misfolded SOD1 by 23% in the spinal cord and modestly prolonged the lifespan of G93A-SOD1 mice [188]. An active immunization strategy targeting monomer/misfolded SOD1 has also been utilized in an attempt to alleviate the burden of pathological SOD1 in neuronal tissue. An antigenic peptide based on the epitope recognized by the SOD1 exposed dimer interface (SEDI) antibody [48] was introduced into G93A-SOD1 or G37R-SOD1 transgenic mice *via* intraperitoneal injection beginning at 6 weeks of age, followed by a series of booster injections, resulting in delayed disease onset, extended disease duration and lifespan, and decreased levels of misfolded conformers of SOD1 in the spinal cord [189]. This study reinforces the key role of misfolded SOD1 in

SOD1-linked ALS pathogenesis and demonstrates the potential of an active immunization approach for the treatment of ALS.

In addition to immunotherapeutic approaches, attempts at targeting misfolded SOD1 through gene therapy have also been experimented with. Adeno-associated virus (AAV)-mediated expression of a DNA construct encoding a secretable single-chain fragment variable antibody, composed of the variable heavy and light chain regions of a monoclonal antibody that specifically recognizes misfolded SOD1, was used to treat G93A-SOD1 transgenic mice, resulting in reduced neuronal stress signals and a decreased load of misfolded SOD1 in spinal cord tissue [190]. Analogous therapeutic strategies have yet to be attempted for other ALS-associated proteins, although other methodologies have been used to target TDP-43 toxicity. For example, suppressor screens in yeast have identified a gene, *DBR1*, which when deleted supresses TDP-43 pathology in yeast and human cells [191], providing a therapeutic target that specifically effects pathological TDP-43, a disease marker in the majority of sporadic ALS cases. Needless to say, exploitation of the prion-like properties of ALS-associated proteins is a *bona fide* area of therapeutic study that will hopefully fill the void of effective treatments that are so desperately needed by the ALS patient community.

CONCLUSION

Protein misfolding and unfolding is a common occurrence in cells subject to the stresses of a disease state. However, most non-native protein conformations generally do not acquire prion-like properties, thus prions represent a unique type of pathogen. As we learn more about the properties of 'what makes a prion a prion', researchers of neurodegenerative proteinopathies continually demonstrate that the prion paradigm is more common than once thought, extending from the once-mysterious livestock diseases of 'mad cow' and scrapie, and the rare instances of human spongiform encephalopathy (e.g., Creutzfeldt–Jakob disease) to relatively common neurodegenerative diseases of aging such as Alzheimer and Parkinson diseases. Although the uniqueness of prions may be diminished, their biological relevance has never been greater. That being said, to date there is no published experimental evidence demonstrating that any proteinopathy, other than the classical prion diseases, can spread from individual to individual in humans or experimental model systems *via* natural infection pathways such as oral or intravenous inoculation. A recent study examining the use of human growth hormone derived from the cadavers of Alzheimer and Parkinson disease patients reaffirms that person-to-person transmissibility for these diseases is highly unlikely to occur [192]. However, one cannot ignore the more demonstrative observations of facilitated trans-organism disease transmission when intraperitoneal inoculation of misfolded amyloid-beta-enriched brain extract from Alzheimer's patients into transgenic mice induces amyloidosis in the brain, a region considerably non-local to the site of injection [193]. Thus, it is likely that the point of entry of prion-like proteins is crucial for even the slightest chance of interorganismal transmission to occur, if biologically permitted. In the single example of animal-to-animal transmission of motor neuron disease presented in the literature to-date, 'seed' in the form of spinal cord homogenate from pathological G93A-SOD1 mice was introduced *via* spinal and intracerebroventricular injections into G85R-SOD1:YFP transgenic mice within 24 h of birth [32], hardly natural circumstances for infection, but the experiment

does show a successful proof-of-concept that, under the right experimental conditions, motor neuron disease can be transmissible. This will no doubt be the first of many subsequent studies investigating *in vivo* transmission of ALS pathology, an important step in understanding the mechanisms of disease progression, which can expectantly lead to therapeutic exploitation. Neurodegenerative diseases of aging, including ALS, represent a dire health concern for developed nations as human lifespans continue to increase due to improving health care and technology. With advancing age comes greater dysfunction of intracellular repair and scavenging mechanisms, contributing to the accumulation of misfolded proteins and the potential seeding of a propagating process [194, 195, 196]. Exploitation of the prion characteristics of ALS may provide the best answer yet to stopping the unrelenting spread of this disease, and other neurodegenerative syndromes.

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