From molecule to molecule and cell to cell: Prion-like mechanisms in amyotrophic lateral sclerosis

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Abstract

Prions, self-proliferating infectious agents consisting of misfolded protein, are most often associated with aggressive neurodegenerative diseases in animals and humans. Akin to the contiguous spread of a living pathogen, the prion paradigm provides a mechanism by which a mutant or wild-type misfolded protein can dominate pathogenesis through self-propagating protein misfolding, and subsequently spread from region to region through the central nervous system. The prion diseases, along with more common neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and the tauopathies belong to a larger group of protein misfolding disorders termed proteinopathies that feature aberrant misfolding and aggregation of specific proteins. Amyotrophic lateral sclerosis (ALS), a lethal disease characterized by progressive degeneration of motor neurons is currently understood as a classical proteinopathy; the disease is typified by the formation of inclusions consisting of aggregated protein within motor neurons that contribute to neurotoxicity. It is well established that misfolded/aggregated proteins such as SOD1 and TDP-43 contribute to the toxicity of motor neurons and play a prominent role in the pathology of ALS. Recent work has identified propagated protein misfolding properties in both mutant and wild-type SOD1, and to a lesser extent TDP-43, which may provide the molecular basis for the clinically observed contiguous spread of the disease through the neuroaxis. In this review we examine the current state of knowledge regarding the prion-like properties of proteins associated with ALS pathology as well as their possible mechanisms of transmission.

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Introduction

The identification of prions, or infectious proteins, over thirty years ago (Diener et al., 1982; Prusiner, 1982) as the transmissible agent responsible for a myriad of brain wasting diseases such as scrapie in
sheep, bovine spongiform encephalopathy (BSE), also known as mad cow disease in cattle, and the devastating human diseases kuru and Creutzfeldt–Jakob disease (CJD), among others was a landmark development in the fields of infectious disease and protein biochemistry. The transmission of disease between organisms without the requirement of nucleic acid was deemed heretical for its time, and even now the concept faces occasional bouts of controversy. Since then, the singularity of pathological mammalian prion protein (PrP) as the sole example of a true infectious protein has come under constant challenge; from earlier studies in yeast (Patino et al., 1996) and other fungi (Uptain and Lindquist, 2002) to more recent work in human neurodegenerative diseases (Aguzzi, 2009; Guest et al., 2011). As such, aberrant protein misfolding and aggregation are a part of the pathobiology of numerous relatively common neurodegenerative disorders including Parkinson’s disease (PD), the tauopathies and Alzheimer’s disease (AD), known collectively as proteinopathies. Specific proteins in these diseases have been identified that appear to emulate the propagating protein mechanism of prions: amyloid-β (Aβ) in AD (Eisele et al., 2010; Meyer-Luehmann et al., 2006), α-synuclein in PD (Luk et al., 2012, 2009), and tau protein in AD, frontotemporal dementia (FTD) and other dementias (Clavaguera et al., 2009; Frost et al., 2009; Guo and Lee, 2011).

The level of experimental evidence describing the prion-like activities implicated in these diseases varies considerably. While evidence of a prion-like mechanism in AD and PD has populated the literature for over a decade, a relative newcomer to the prion paradigm is amyotrophic lateral sclerosis (ALS). When one examines the clinical pathology and epidemiology of ALS, its inclusion into the family of prion-like proteinopathies is quite reasonable. For example, the most significant clinical feature of ALS is its relentless, but contiguous spread through the nervous system; pathology starts at one or more focal points (Sekiguchi et al., 2014) of onset, possibly beginning in cortical neurons (Braak et al., 2013), and spreads in a spatiotemporal fashion through adjacent neuroanatomical regions (Ravits et al., 2007; Ravits and La Spada, 2009). Given the similarities in progression and anatomical spread among neurodegenerative proteinopathies, including the classical prion diseases, a likely mechanism to account for this observation that has gained traction in recent years is the region to region prion-like spread of propagated protein misfolding. In addition, this mechanism also accounts for how a mutant or even wild-type protein can dominate pathogenesis of a phenotypically diverse disease such as ALS, akin to when the normal cellular isoform of prion protein (PrPc) converts to its pathologically dominant form PrPSc in prion disease. To date at least two proteins associated with the pathobiology of ALS have demonstrated multiple aspects of prion-like activity: SOD1 and TDP-43. This review attempts to summarize the known experimental evidence for prion-like activity of these proteins, and comment on their possible mechanisms of intercellular spread.

An introduction to ALS

ALS, also known as Lou Gehrig’s disease in the United States, is a rapidly progressive fatal neuromuscular condition characterized by degeneration of the upper and lower motor neurons causing progressive paralysis and spasticity that affects the muscles of mobility, speech, swallowing and respiration (Bradley, 2009; Cleveland and Rothstein, 2001), although cognitive function is usually spared with only 5% of patients developing frank frontotemporal dementia (FTD) (Phukan et al., 2007). It is the most common form of motor neuron disease worldwide and has 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 (Strong and Rosenfeld, 2003). The etiology of ALS is unknown; however, similar to other neurodegenerative diseases such as AD and CJD, the disease can be divided into two categories, sporadic and familial. 90–95% of ALS cases are sporadic (SALS) whereas only some predisposing gene mutations have been identified, such as the ataxin-2 repeat expansions (Elden et al., 2010). The remainder of cases are familial (FALS) (Haverkamp et al., 1995), which are predominantly associated with Mendelian-inherited 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 (ALS Mutation Database, 2007; Deng et al., 2011; Stewart et al., 2012; Wu et al., 2012). Clinically, both categories are very similar suggesting that a common downstream pathogenic mechanism, regardless of disease origin, may lie at the heart of the disease (Kabashi et al., 2007).

ALS is considered a protein misfolding disorder, based on its neuropathology, and as such is classified as a proteinopathy, similar to other neurodegenerative diseases. 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 by astrocyte activation and proliferation of microglia (Philips and Robberecht, 2011). 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 the aggregation of misfolded protein. Protein misfolding can be triggered by a multitude of factors, including genetic mutation, oxidation, aberrant post-translational modification, dysfunctional proteostasis and seeded polymerization. In FALS cases where a SOD1 mutation is identified, the primary component of these protein inclusions is SOD1 itself (Kato et al., 2000). In sporadic cases where SOD1 mutations have been excluded there is recent evidence supporting the presence of misfolded SOD1-containing inclusions as well (Bosco et al., 2010; Forsberg et al., 2010; Grad et al., 2014; Pokrishevsky et al., 2012), although this observation is not one of consensus (Kerman et al., 2010). A more universally agreed observation is that 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 (Neumann et al., 2006).

Pathological SOD1 in ALS

Mutations in the gene encoding SOD1, a ubiquitously-expressed free-radical scavenging enzyme, were the first genetic cause of ALS to be identified (Rosen, 1993) and are implicated in ~20% of all FALS cases. The primary function of SOD1 is the conversion of the highly toxic free-radical superoxide to water and hydrogen peroxide (Beckman and Koppenol, 1996). To date, over 150 different disease-causing SOD1 mutations have been identified (ALS Mutation Database, 2007; Andersen et al., 2003). Despite the intrinsic stability of the native SOD1 enzyme, the majority of these mutations induce misfolding and subsequent aggregation. 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 oligomeric structures (Rakhit et al., 2004, 2007). However, 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 (Casoni et al., 2005; Rakhit et al., 2004, 2002) in a concentration-dependent manner (Rakhit et al., 2004). There is increasing evidence that all types of ALS, including non-SOD1-linked familial and sporadic cases are associated with SOD1 misfolding, oxidation and aggregation (Matias-Guiu et al., 2008; Synofzik et al., 2012). Inclusions containing aggregated SOD1 have been detected in spinal cord tissues from both FALS and SALS patients (Chou et al., 1996a, 1996b; Shibata et al., 1994) in addition to biochemical, genetic and immunological evidence of misfolded SOD1 in cases of SOD1-excluded SALS (Bosco et al., 2010; Broom et al., 2008; Forsberg et al., 2010; Grad et al., 2014; Cruzman et al., 2007; Pokrishhevsky et al., 2012). Misfolded SOD1 is therefore a prime candidate for a primary role in the pathogenesis of ALS.
candidate as a common molecular determinant for all forms of ALS and may play a key role in disease pathogenesis.

Mutations in SOD1 can impair enzymatic function; however loss of superoxide dismutase function is not the cause of disease as SOD1 knockout mice do not develop an ALS-like motor neuron disease. Conversely, a mutant SOD1 transgenic mouse developed a progressive motor neuron disease despite increased SOD1 enzymatic activity over and above the mouse endogenous protein (Gurney et al., 1994). Instead, misfolding of SOD1 conferred by mutation, oxidation or other cell stresses is generally thought to acquire a toxic gain of function (Andersen et al., 1995; Gurney et al., 1994; Reaume et al., 1996), though the precise nature of this toxicity and its specificity for motor neurons remain to be elucidated. The idea that misfolded/aggregated SOD1 directly contributes to neurotoxicity is lent credence by the observation that all transgenic mouse strains expressing ALS-causing SOD1 mutants develop large intracellular cytosolic inclusions in motor neurons and accessory neural cells that coincide with the onset of motor neuron disease and increase throughout the course of disease progression (Brujin et al., 1997; Cleveland, 1999). Moreover, in its misfolded pathological form SOD1 can react non-specifically with a variety of substrates, causing it to actually become a net producer of reactive oxygen and nitrogen species (Said Ahmed et al., 2000), as opposed to a free-radical scavenger, which can subsequently lead to intracellular damage of cellular protein, lipid, and nucleic acid (Beckman et al., 1993, 2001). 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 (Bard et al., 2012; Bendotti et al., 2012; Cleveland and Liu, 2000).

Intermolecular conversion of SOD1

Central to the prion schema is the ‘protein-only’ hypothesis that stipulates that misfolded protein alone is necessary and sufficient to induce further misfolding in native protein, without the requirement of nucleic acids or other co-factors (Prusiner, 1982). That is to say, one molecule can literally impart its misfold on a neighbouring molecule. Although the depth of experimental evidence is not as established compared to the prion protein, an ever-growing body of evidence in the literature suggests that misfolded SOD1 has the ability to transfer conformational information from one molecule to another, meeting at least the intermolecular requirements of prion-like activity (Chia et al., 2010; Grad et al., 2011; Munch et al., 2011). SOD1, a small soluble ubiquitously-expressed 153-residue protein, exists as a homodimer in its normal enzymatically active form. The native holo-enzyme contains an intramolecular disulphide bond within each monomer, which contributes to its high conformational stability and resistance to proteolytic digestion (Ratovitski et al., 1999). Despite this, SOD1 is highly prone to destabilization when mutated or aberrantly oxidized. In its mutated or post-translationally modified state SOD1 has a high propensity to misfold and form multimERIC species and aggregates. Under denaturing conditions wild-type and mutant forms of SOD1 can spontaneously form aggregates and fibrils in vitro (Chia et al., 2010), where the relative propensity for aggregation is dependent upon the SOD1 mutant variant (Prudencio et al., 2009b). Indications of in vivo SOD1 fibril formation have been observed in transgenic mice expressing mutant SOD1 (Furukawa et al., 2008; Wang et al., 2002). 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 (Kerman et al., 2010).

Intramolecular conversion of native WTSOD1 to a pathological form has been indirectly observed in a series of in vivo studies. Acceleration of motor neuron degeneration in mutant SOD1 mouse models is observed upon co-expression of human WTSOD1 (Jaarsma et al., 2000; Wang et al., 2009), possibly through intermolecular stabilization of mutant species via WTSOD1 interactions (Fukada et al., 2001) or via formation of non-native disulphide interactions that induce insoluble aggregate formation (Deng et al., 2006). Co-aggregation of mutant and WTSOD1 has also been detected in tissue derived from familial ALS patients (Brujin et al., 1998), suggestive of intermolecular conversion in the pathology of the disease in patients. Direct evidence of intermolecular WTSOD1 conversion to a misfolded isoform has been observed in mesenchymal and neuron-like human cell lines (Grad et al., 2011; Pokrishavskiy et al., 2012). Expression of misfolded human SOD1 mutants can convert endogenous WTSOD1 to a misfolded form, revealed by conformation-specific antibodies that recognize epitopes only accessible when the protein is in a misfolded state. The induced misfolding by mutant SOD1 results in enhanced protease sensitivity, which is interpreted as an indicator of global loosening of the polypeptide backbone. Conversion of WTSOD1 by a mutant conformer 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 (Grad et al., 2011) thus further supporting the ‘protein-only’ paradigm. Interestingly, intermolecular conversion of WTSOD1 by a misfolded template 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 (Raymond et al., 2000). Conversion of human WTSOD1 to a misfolded form is restricted to the exposure of a single amino acid residue, a tryptophan (Trp) at position 32 (Grad et al., 2011), indicating a potential point of contact between the converting and 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 (Taylor et al., 2007). SOD1 constructs featuring ALS-causing mutations, but with a serine substitution at position 32 failed to induce WTSOD1 misfolding in human cell lines (Grad et al., 2011). Furthermore, mouse endogenous SOD1, which inherently possesses a serine at position 32, is also inert to conformational conversion of human SOD1 in vitro (Grad et al., 2011). In vivo, human WTSOD1 does not accelerate motor neuron disease in mice expressing mutant murine SOD1 with a G86R substitution (Audet et al., 2010), analogous to the human G85R mutation linked to FALS. Furthermore, murine SOD1 is not incorporated into aggregates of human mutant SOD1 (Deng et al., 2006; Prudencio et al., 2009a; Wang et al., 2009). Given the collective evidence, SOD1 satisfies the intermolecular characteristics of prion-like protein conversion that adheres to sequence/structure specificity reminiscent of the species barrier observed for certain prion diseases.

Mechanisms of propagated SOD1 misfolding

Another key characteristic of the prion paradigm is the ability for newly misfolded protein substrate to provide new template for additional rounds of misfolding. Two mechanisms have been proposed to account for this conversion process: nucleated polymerization, in which the misfolded species is intrinsically less stable as a monomer but becomes more stable than its native counterpart when recruited to a multimeric aggregate; and template-directed misfolding, in which the misfolded pathological conformer is more stable than the native form but is kinetically inaccessible without catalysis by interaction with the misfolded form (Horwich and Weissman, 1997). Evidence for both template-directed misfolding and nucleated polymerization have been observed for SOD1 and suggests that propagated misfolding of both mutant and WTSOD1 spans a continuum between the two models. In vivo surface plasmon resonance experiments with natively folded WTSOD1 suggest that the intracellular conversion of WTSOD1 likely occurs in a template-directed fashion (Grad et al., 2011). Given the intrinsic stability of homodimeric native SOD1, and that co-expression of

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WTSOD1 has been observed to stabilize mutant misfolded SOD1 (Prudencio et al., 2010; Witan et al., 2009), it is possible that nascent WTSOD1 polypeptide newly-emerged from the cell’s translation machinery may provide a thermodynamically favourable substrate for seeded polymerization to occur (Fig. 1), although this remains to be experimentally verified.

Propagated misfolding between molecules of mutant SOD1, which are already prone to aggregate, likely use a combination of template-directed misfolding and seeded polymerization, depending on the intrinsic aggregation properties and structural stabilities of different mutants, which plays an essential role in disease progression and patient survival (Wang et al., 2008). 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 (Elam et al., 2003) suggesting that this conformational rearrangement in the metal-deficient enzyme could contribute to SOD1-linked FALS pathology. For nucleated polymerization to occur, a pre-formed high-order structure acts as a nucleus for soluble protein to come into contact with. The soluble protein subsequently becomes incorporated into the aggregate via conformational change. Recombinant apo-SOD1 without disulphide bonds, ALS mutant SOD1 protein or insoluble SOD1-containing aggregates isolated from transgenic mouse models of ALS expressing mutant SOD1 have all been shown to possess spontaneous fibrilization and seeding activity in vitro under various non-physiological conditions (Chia et al., 2010; Furukawa et al., 2008). Recent observations suggest that protein to protein conversion induced by both mutant (Munch et al., 2011) and over-expressed WTSOD1 (Grad et al., 2011) can be self-sustaining. Induced misfolding of SOD1 can persist even in the absence of the misfolded seed in cell culture, suggesting that newly misfolded SOD1 can act as template for subsequent cycles of misfolding (Grad et al., 2014). Exogenously-applied aggregated mutant human SOD1 can induce subsequent aggregation of soluble transgenically-expressed mutant SOD1 in mouse neuroblastoma cells (Munch et al., 2011). 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 (Furukawa et al., 2013).

**Intercellular transmission of SOD1 misfolding**

It is clear that 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 has no way to effectively transmit its misfold to other anatomical regions. Indeed, intercellular transmission of propagated misfolding and aggregation of pathogenic PrPSc is a hallmark of the prion diseases. As mentioned before, release and uptake of protein aggregates or proto-fibrils is one possible mechanism for intercellular spread in ALS, and likely occurs when dying neurons release their contents to the extracellular environment. For example, exogenous mutant (Munch et al., 2011) and WTSOD1 (Grad et al., 2014) 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 (Guest et al., 2011) that can better survive the relative hostilities of the extracellular environment than lower-order soluble forms.

Although formation of large protein aggregates and fibrils is an observational pathological characteristic of ALS, there is a question as to whether these protein structures or other forms are responsible for neurotoxicity. 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 (Haass and Selkoe, 2007). In Alzheimer’s disease, it is smaller oligomeric forms of amyloid-β that impair synaptic function (Kayed et al., 2003; Lesne et al., 2006), plasticity and memory (Shankar et al., 2008). Likewise, there is growing evidence that pre-fibrillar oligomers of α-synuclein are responsible for disease progression in Parkinson’s disease (El-Agnaf et al., 2006; Lashuel et al., 2002) as opposed to the hallmark Lewy bodies, which may simply serve as benign “dumps” of aggregated protein. It is reasonable that a similar paradigm exists for SOD1 misfolding in ALS, which could explain why misfolded SOD1 is detectable in sporadic cases, but not the large protein inclusions usually observed in mutant SOD1-associated familial ALS (Bosco et al., 2010; Forsberg et al., 2010; Grad et al., 2014) insinuating that misfolded SOD1 in SALS is present as smaller soluble species not readily detectable by gross histochemical staining of tissue sections. Before neuronal cell death occurs in ALS, pathology spreads from region to region in a spatiotemporal manner (Ravits and La Spada, 2009) suggesting an active cellular process that may be more relevant to the early stages of the disease. One mechanism by which this can occur is through small 30–80 nm-wide membrane-bounded vesicles, called exosomes, which normally transport various cargo such as proteins and nucleic acids between cells. Exosomes have been increasingly implicated in the spread of misfolded or aggregated proteins involved in neurodegenerative diseases, such as Alzheimer’s disease (Perez-Gonzalez et al., 2012), Parkinson’s disease (Danzer et al., 2012; Emmanouilidou et al., 2010) and the prion diseases (Coleman et al., 2012; Fevrier et al., 2004). The evidence supporting an analogous mechanism involved in ALS is ever-increasing. Exosomes are formed within intracellular endosomes by membrane invagination, resulting in the formation of multivesicular bodies (MVBs). MVBs are normally involved in the lysosome 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 (Keller et al., 2006). SOD1 itself is a known resident protein of exosomes from multiple cell types and species (Kim et al., 2013). Subsequent studies have identified exosomes as the secretion mechanism for both wild-type and mutant SOD1 in a motor neuron-like cell model (Gomes et al., 2007), potentially mediated by chromogranins (Urushitani et al., 2006), acidic components of neurotransmitter-enriched secretory vesicles (Huttner et al., 1996) that could serve as a secretory chaperone. Further evidence suggests that mutant SOD1 oligomers accumulate in the endoplasmic reticulum–Golgi compartments of the endocytic pathway prior to its subsequent secretion (Urushitani et al., 2008). More recent work has established that both mutant and wild-type misfolded SOD1 can be secreted from neuron-like cells via exosomes (Basso et al., 2013); these exosomes can then be subsequently taken up by fresh cells where the misfolded SOD1 cargo provides a template for subsequent induction of protein misfolding (Grad et al., 2014) (Fig. 1). Misfolded SOD1 is immunologically detectable on the outer leaflet of extracellular vesicle membranes (Grad et al., 2014) and is therefore accessible for targeting by therapeutic molecules in the extracellular milieu. More recently, mouse astrocyte-derived exosomes were observed to efficiently transfer mutant SOD1 to spinal neurons and subsequently cause selective motor neuron death (Basso et al.,

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**Fig. 2.** Pathways of pathological TDP-43. TDP-43 translocation from the nucleus to the cytoplasm occurs either via mutation or cell stressors (1). Cytoplasmic TDP-43 has a propensity to aggregate (2) via its C-terminal fragment (CTF), forming nucleating seeds that recruit other soluble TDP-43 molecules into larger aggregates (3). Similarly, TDP-43 can be proteolytically cleaved, releasing its CTF, which can form aggregates with other CTFs via its prion-like domain, forming inclusions (4). 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 (5). Conversely, TDP-43 aggregate uptake (6) has also been observed, but the mechanism by which it occurs has yet to be identified.

**Legend:**
- $\Delta$ = TDP-43 monomer
- $\Delta$ = TDP-43 C-terminal fragment

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Seeded aggregation of TDP-43 (summarized in Fig. 2). Inclusions, TDP-43 clearly has strong potential for prion-like behavior like domain and the presence of C-terminal fragments within neuronal inclusions, TDP-43 clearly has strong potential for prion-like behavior. On the basis of its C-terminal prion domain into the C-terminus of TDP-43 successfully recapitulate the cytoplasmic mislocalization and toxicity seen in disease. These models show that the prion-like domain of TDP-43 is essential for normal protein function as demonstrated in vivo (Wang et al., 2012). Nevertheless, 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 (Furukawa et al., 2011). These induced aggregates are detergent-insoluble and ubiquitinated, similar to that observed for pathological TDP-43 in ALS patients, suggesting that seeded aggregation of TDP-43 is an important aspect of disease pathology. Interestingly, seeded aggregation is a phenomenon observed for several proteinopathies, including the prion diseases (Aguzzi and Rajendran, 2009), Alzheimer’s disease (Hardy and Selkoe, 2002; Meyer-Luehmann et al., 2006) and Parkinson’s disease (Lu et al., 2009).

In further support of the prion paradigm, self-templating properties have also been observed for TDP-43. 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 (Nonaka et al., 2013). 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. However, it is unclear if induced aggregation persists even in the absence of the original templating seed, indicative of an authentic prion-like self-propagating mechanism. Most importantly, the induced intracellular aggregates of TDP-43 were toxic to cultured neuroblastoma cells, possibly via a mechanism involving proteasome dysfunction (Nonaka et al., 2013), establishing a solid link between propagated TDP-43 misfolding and disease pathology. It should be noted that possible cell-to-cell spread has also been observed for propagated TDP-43 aggregation as phosphorylated aggregates of TDP-43 expressed in cultured neuroblastomas have been shown to induce TDP-43 aggregation in cells that did not originally contain them (Nonaka et al., 2013). Furthermore, TDP-43 pathology in ALS is clinically observed to spread in a sequential pattern through the neuroaxis in agreement with the aforementioned cell biology data, and consistent with a requisite behaviour for the emerging prion paradigm for this protein (Brett Schneider et al., 2013). It should be noted that exosome-mediated transmission may not be specific to just SOD1 in ALS. Possible evidence for exosome involvement in TDP-43 intercellular transmission has also been observed recently. Full-length TDP-43 is enriched in exosome fractions isolated from human neuroblastoma cells treated with seed from ALS patient extract (Nonaka et al., 2013).

**Prion-like activity in other proteins associated with ALS**

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 pathology. Fused in sarcoma (FUS), Ewing sarcoma breakpoint region 1 (EWSR), and TATA-binding protein associated factor 15 (TAF15) are three such proteins which share similar domain architecture and together form the TET family of proteins (reviewed in [Tan and Manley, 2009]). FUS mutations causing ALS were first identified in 2009 and were shown to cause mislocalization of the protein from the nucleus to the cytoplasm and inclusion formation similar to TDP-43 (Kwiatkowski et al., 2009; Vance et al., 2009). Likewise, expression of mutant FUS in yeast causes...
aggregation and cytotoxicity (Gitler and Shorter, 2011). Like TDP-43, FUS is an RNA-processing enzyme and contains a prion-like domain; however the FUS prion-like domain is located in the N-terminus of the protein. The minimal protein region of FUS required for aggregation is larger than in TDP-43; in FUS, the prion-like domain alone is insufficient to cause aggregation and requires the RNA-binding RRM and glycine-rich RGG domains (Sun et al., 2011). Interestingly, 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 (Gitler and Shorter, 2011). FUS has different binding partners than TDP-43, and the two do not co-localize within pathological inclusions in the cytoplasmic (Huang et al., 2010; Sun et al., 2011) despite their structural, functional and pathological similarities suggesting that the two proteins are involved in pathology via distinct, albeit related, mechanisms. 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 (Couthouis et al., 2011). Like other TET family members, TAF15 has a QGSY-rich N-terminal region which is also predicted to be a prion-like domain (King et al., 2012). 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 (Thomsen et al., 2013). 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 (Couthouis et al., 2011). 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 (Thomsen et al., 2013). However, evidence of self-propagation of protein misfolding or intercellular transmission of these proteins is yet to be presented.

Can ALS be considered a true prion disease?

The cell to cell transmission of propagated protein misfolding now appears to be a common mechanism in neurodegenerative diseases of aging; however, the question remains whether ALS or any of the other non-prion protein neurodegenerative diseases mentioned in this review can be truly considered as authentic prion diseases. One would argue that despite fulfilling certain required characteristics of pathological prion proteins, such as evidence of intermolecular protein recruitment and conversion, intercellular transmission, and neuroanatomical migration, SOD1 and other analogous prion-like proteins involved in neurodegenerative proteinopathies do not share key traits of the classical prion diseases, namely transmission between organisms (Guest et al., 2011) and the evidence of strains that vary in their infectivity and toxicity (Legname et al., 2005). Moreover, there is little evidence in the literature to date that resolutely confirms the protein-only hypothesis in any of the other non-classical prion diseases in a reductionist in vitro system; the lone exception may be for SOD1 as template-directed misfolding was observed in a completely cell-free environment using purified recombinant protein (Grad et al., 2011).

When it comes to organisinal infectivity no other pathologically misfolded protein comes close to the virulence of PrPSc in prion disease. To date, there is no published experimental evidence demonstrating that any neurodegenerative 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 AD and PD patients reaffirms that person-to-person transmissibility for Alzheimer’s and Parkinson’s diseases is highly unlikely to occur (Irwin et al., 2013). 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 (Eisele et al., 2010). It is therefore likely that the point of entry of other prion-like agents is crucial for disease propagation, indicating a limited mode of transmission compared to PrPSc, the prototypical highly-infectious prion. Sensitivity to the extracellular environment may explain the lack of evidence thus far for organism-to-organism spread of ALS, at least via misfolded SOD1. Unlike PrPSc, which is a highly-stable and protease-resistant isoform compared to normal PrP, and represents the epitome of a transmissible pathological protein-only agent, many pathological species of misfolded SOD1 become increasingly protease-sensitive and unstable (Grad et al., 2011; Ratovitski et al., 1999). This would make a misfolded SOD1 transmissible particle less likely to stand up to the environmental rigours of person-to-person transmission, although the presence of native wild-type SOD1 has been shown to increase the stability of mutant misfolded SOD1 (Weichert et al., 2013). Other factors that may confound interorganism infection of SOD1 or TDP-43 misfolding/aggregation include misfolding kinetics, i.e. it simply takes too long for suitable levels of misfolded template to replicate to levels that facilitate infection, and host attributes that may simply be incompatible for misfolded proteins of ALS to transmit between individuals. However, SOD1 does appear to meet the intermolecular and intercellular requirements associated with prions, and TDP-43 clearly has the ability to recruit soluble monomer into larger insoluble aggregates. Taken together, the literature suggests that the pathological agents of neurodegenerative proteinopathies could all be considered prions on a molecular and cellular scale. However, for prion proteins in general transmissibility and toxicity characteristics fall within a wide spectrum, with PrPSc representing the extreme end (highly transmissible, highly toxic) and proteins associated with more relatively common neurodegenerative proteinopathies, such as SOD1, TDP-43, amyloid-β or α-synuclein, grouped near the other end as less robust transmissible agents. There is no doubt that further in vivo characterization of misfolded protein transmissibility in ALS is required to determine the risks, if any, for interorganism transmission, and how much of a role misfolded protein propagation plays in motor neuron disease progression and pathology. Needless to say, the uniqueness of prions may have somewhat diminished in the past decade, but the importance in understanding their biology with respect to more common proteinopathies has never been greater; the significance of which is even higher for a disease like ALS, which lacks any type of intervention that significantly delays disease onset or slows progression. Targeting the prion-like mechanisms of this disease may provide much needed hope for an effective intervention.

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