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Review

Prions and the proteasome

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ABSTRACT

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy in animals. They are unique in terms of their biology because they are caused by the conformational re-arrangement of a normal host-encoded prion protein, PrP^C, to an abnormal infectious isoform, PrP^{Sc}. Currently the precise mechanism behind prion-mediated neurodegeneration remains unclear. It is hypothesised than an unknown toxic gain of function of PrP^{Sc}, or an intermediate oligomeric form, underlies neuronal death. Increasing evidence suggests a role for the ubiquitin proteasome system (UPS) in prion disease. Both wild-type PrP^C and disease-associated PrP isoforms accumulate in cells after proteasome inhibition leading to increased cell death, and abnormal β-sheet-rich PrP isoforms have been shown to inhibit the catalytic activity of the proteasome. Here we review potential interactions between prions and the proteasome outlining how the UPS may be implicated in prion-mediated neurodegeneration.

1. Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a family of fatal neurodegenerative disorders (Table 1). They affect both humans and animals, and can be transmitted within or between animal species by inoculation or dietary exposure [1]. Despite their relative rarity–it is estimated that they affect one person per million worldwide annually–they remain in the spotlight due to the unique biology of the transmissible agent. Their pathogenesis is associated with a conformational re-arrangement of a normal cellular prion protein, PrP^C, to an abnormal isoform, PrP^{Sc} (Sc for scrapie) [2]. Prion diseases share the following histopathological hallmarks: spongiform vacuolation, severe neuronal loss, astrocytic and microglial proliferation, and accumulation of the disease-associated isoform of the prion protein in the brain, sometimes with the formation of amyloid deposits. Currently, the exact cause of prion-mediated neurodegeneration remains unclear and a major gap exists in the understanding of how the conversion of PrP^C to PrP^{Sc} ultimately kills neurons. Here we give an overview of the biology of prion disease, highlighting the mechanisms by which prions might cause neurotoxicity and considering the role of cellular protein degradation systems such as the ubiquitin proteasome system (UPS) in prion-mediated cellular neurodegeneration.

1.1. Prion diseases in humans and animals

The prototypic prion disease is scrapie, a common disease affecting sheep and goats (Table 1). Other mammalian prion diseases include transmissible mink encephalopathy, chronic wasting disease of deer and elk, and bovine spongiform encephalopathy (BSE) in cattle. The latter, which is often referred to as 'mad cow disease', emerged in the UK in 1986, causing a major epidemic thought to have started due to contaminated feed containing ruminant-derived protein [3].

Human prion diseases include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru [4,5] (Table 1). Clinical features comprise rapidly progressive dementia accompanied by cerebellar ataxia, myoclonus and pyramidal or extrapyramidal disturbances. Prion diseases are unique in that they may arise spontaneously, be genetically inherited, or acquired via infection. Approximately 85% of CJD cases occur sporadically with no known cause, whereas 15% of human prion diseases (including GSS, familial CJD and FFI) represent autosomal dominantly inherited conditions associated with mutations in the prion protein gene, PRNP [6]. Acquired forms of the disease include iatrogenic CJD resulting from accidental medical exposure to prions, and kuru, which emerged as an epidemic in the Eastern Highlands of Papua New Guinea and affected mostly women and children involved in ritual cannibalistic feasts [7]. Variant CJD (vCJD), a new acquired human prion disease, first emerged in the mid 1990's in the UK [8] and has affected ~170 individuals (http://www.cjd.ed.ac.uk/figures.htm). Suspicions of a link between vCJD and BSE were raised because they arose at the same time. Clinical, neuropathological and molecular

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Table 1
Prion diseases of humans and animals

Prion disease	Host	Aetiology
Scrapie	Sheep and goats	Ingestion, horizontal transmission
Transmissible mink encephalopathy	Mink	Ingestion
Chronic wasting disease	Mule deer, white-tailed deer, Rocky Mountain elk and moose	Ingestion, horizontal transmission
Bovine spongiform encephalopathy	Cattle	
Feline spongiform encephalopathy	Domestic and captive wild cats	Acquired by ingestion of BSE-contaminated meat
Exotic ungulate encephalopathy	Nyala, oryx and greater kudu	
Sporadic CJD	Human	Unknown
Iatrogenic CJD	Human	Acquired by accidental exposure from medical contact with human prion-contaminated neurosurgical instruments, tissue grafts and pituitary hormones derived from human cadavers
Familial CJD	Human	
Fatal familial insomnia	Human	Inherited; all associated with PRNP-coding mutations
Gerstmann–Sträussler–Scheinker disease	Human	
Variant CJD	Human	Environmental exposure possibly via ingestion of BSE-contaminated meat. Three blood transfusion-associated cases (from vCJD donors)
Kuru	Human	Exposure via endocannibalism in a small area of Papua New Guinea

evidence support the fact that vCJD is caused by the same prion isolate as BSE [9–11]. Although the number of confirmed cases is small, the actual figure of subclinically infected individuals remains unknown. All clinical cases of vCJD to date have occurred in individuals homogygous for methionine at codon 129 of the PRNP gene. Prion diseases are associated with long, clinically silent incubation periods, which in human-to-human transmission, such as seen in kuru, can exceed 50 years [7]. As human infection with BSE involves cross-species transmission, it may confer a substantial prolongation of incubation time and thus vCJD still poses a serious threat to public health. This is highlighted by the efficient secondary transmission of infection via blood transfusion [12–14].

1.2. Protein-only hypothesis

The nature of the causative agent in prion disease has been the focus of intense research. Griffith first proposed that transmission of scrapie was via a protein-only mechanism [15], as no experimental data supported the notion that a nucleic acid is associated with prion infection. For example, the transmissible agent is resistant to procedures that modify nucleic acids, such as ultraviolet and ionizing radiation [2]. In 1982, progressive enrichment of scrapie-infected hamster brain homogenates for infectivity led to the isolation of a protease-resistant protein, designated the prion protein (PrP) [16]. The term 'prion' was first coined by Stanley Prusiner to distinguish the infectious agent in prion disease as a pathogen from bacteria and viruses, and refers to a 'small, proteinaceous infectious particle that resists inactivation by procedures which modify nucleic acids' [2]. Following the purification of the protein, the gene encoding PrP was identified [17]. The normal product of the prion protein gene is a 33–35 kDa protein called the cellular prion protein (PrP^C). The disease-associated isoform of the prion protein, PrP^{Sc}, is post-translationally derived from PrP^C [18,19] and has increased β-sheet content [20]. PrP^C and PrP^{Sc} do not differ in amino acid sequence and there are no antibodies that clearly discriminate between these two isoforms. PrP^{Sc} is currently distinguished from PrP^C by its partial proteinase K (PK) resistance and detergent insolubility (Fig. 1A). Despite worldwide research, the exact nature of the infectious agent is still unknown and there is evidence both for and against PrP^{Sc} as the infectious agent. Even though a form of PrP may be infectious, there is some evidence that this form may not be PrP^{Sc}. This is highlighted by the lack of correlation between infectivity and PrP^{Sc} levels [21,22] as well as by the existence of PK-sensitive forms of PrP^{Sc} [23–29].

The protein-only hypothesis, which postulates that prion propagation results from a change in PrP conformation whereby PrP^{Sc} recruits endogenous PrP^C in order to replicate, is now well established. Some of the strongest supporting evidence comes from findings that inherited prion diseases are linked to mutations in the PRNP gene [1], indicating that a genetic disease may be able to propagate in an infectious way. Another important line of evidence comes from PrP^C knockout mice, which are resistant to scrapie prions [30]. Moreover, infectious prions have been shown to consist mainly or exclusively of PrP^{Sc}, as indicated by a large body of experimental data. Most notably, synthetic prions that polymerise into fibrils in vitro have been shown to be infectious in vivo [31]. Similarly, the in vitro generation of prions by the protein-misfolding cyclic amplification assay (PMCA), shows that synthetic prions share similar biochemical characteristics to the disease-associated isoform of the prion protein and when inoculated into mice lead to a scrapie-like disease [32]. However, the existence of multiple prion isolates, or strains, which are characterised by differences in incubation period, biochemical properties, neuropathological and clinical features, have posed problems for the protein-only model for prion propagation. These differences have been previously attributed to different conformation or aggregation states of PrP^{Sc}, each having distinct abilities to catalyse the conversion process [23,33]. Recently, however, a general hypothetical model of prion strains and their pathogenicity has been proposed that explains how multiple prion strains could exist within the protein-only hypothesis criteria [34]. Alternative possibilities such as the 'unified hypothesis', put forward by Charles Weissmann, argue that although infectivity depends only on the protein, strain characteristics could be encoded by a small nucleic acid [35]. A protein-only based mechanism of inheritance may have wider relevance in biology and these mechanisms have been extensively investigated in yeast and fungi [36–38].

1.3. Prion protein

1.3.1. Structure and function

PrP^C is a highly conserved, approximately 250 amino acid glycoprotein, abundant in neuronal and glial cells of the central nervous system (Fig. 1B). It has two *N*-glycosylation sites and is glycosylphosphatidylinositol (GPI)-anchored to the plasma membrane [39]. Its C-terminal domain is folded largely into α-helices stabilised by a single disulphide bond and NMR spectroscopy shows it is monomeric [40]. The N-terminus contains an octapeptide repeat region with tight binding sites for Cu^{2+} [41]. PrP^C is glycosylated in the ER after the removal of an N-terminal signal peptide [42] (Fig. 1B). The GPI anchor is attached after cleavage of a second signal peptide at the C-terminus. The protein is then trafficked via the Golgi to the cell surface, where it is internalised in clathrin-coated pits and endocytosed for recycling [43,44]. The function of PrP^C remains unknown, although a number of roles have been proposed due to its remarkable conservation between species [45]. Loss of PrP^C expression shows no overt phenotype in both embryonic PrP^C-null mice and adult-onset knockout models [46,47]. However, these embryonic PrP^C-null mice develop abnormalities in synaptic physiology, circadian rhythm and

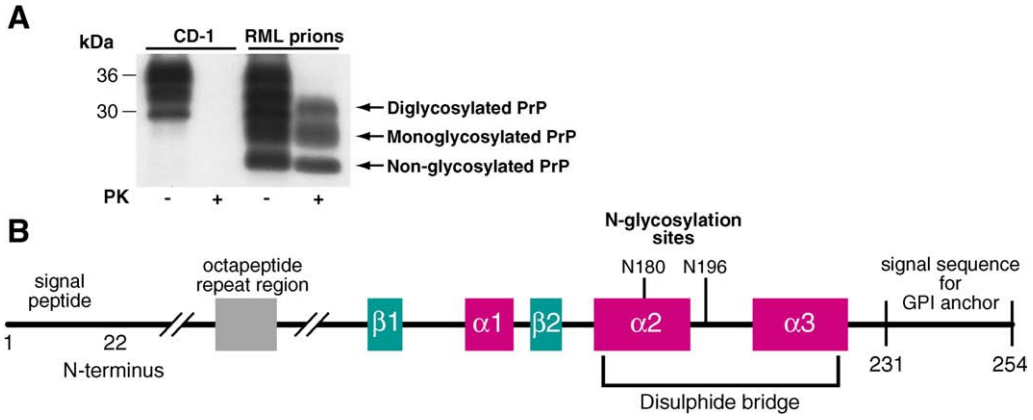

Fig. 1. Prion protein (A) Immunoblot analysis of normal mouse brain (CD1) and prion-infected mouse brain (RML) before and after proteinase K (PK) treatment. PrP^{C} is completely digested by PK treatment in both normal and RML mouse brain. In contrast, PrP^{Sc} present in prion-infected mouse brain is partially resistant to PK treatment, which leads to the generation of amino-terminal-truncated fragments of diglycosylated, monoglycosylated or non-glycosylated PrP. (B) A schematic of the normal cellular murine prion protein.

sleep patterns [48,49]. Putative functions for PrP^{C} are based on its localisation and on PrP^{C}-interacting molecules, and include cell adhesion [50–52], synaptogenesis [53], signalling [54], copper homeostasis [55], and neuroprotection [56–59]. PrP^{C} has also been implicated in the self-renewal of hematopoietic stem cells during serial transplantation [60]. Recently, links between Alzheimer's disease (AD) and prion disease have been proposed as PrP^{C} was shown to regulate neurotoxic Aβ production by inhibiting β-secretase cleavage of the amyloid precursor protein [61].

1.3.2. Prion-mediated toxicity

Prion-mediated cell death appears to be caused via apoptosis [62,63], but the exact mechanism is poorly understood. A full overview of possible pathways of prion-mediated neurotoxicity is beyond the scope of this review but has been recently reviewed [64–66]. PrP^{C} is essential for the development of prion disease, but loss of function of PrP^{C} is unlikely to be the cause of pathology because neither embryonic nor adult knockout of PrP^{C} results in neurodegeneration [46,47]. It is widely hypothesised that an unknown toxic gain of function of PrP^{Sc}, or its precursor, is more likely to underlie cell death. Studies have suggested that both full-length PrP^{Sc} [67] and shorter PrP peptides are toxic to cells in vitro [68], but their relevance to in vivo pathogenesis is under debate. There is strong evidence that PrP^{Sc} itself may not be the toxic entity, but instead a toxic oligomeric PrP intermediate species may be produced during prion conversion [34]. For example, PrP^{C}-null tissue remains healthy and free of pathology when exposed to PrP^{Sc} [69,70], and there is no direct correlation between neuronal loss and PrP^{Sc} plaques in CJD brains [71]. In addition, prion diseases in which PrP^{Sc} is barely detectable have also been described [21,72,73], and subclinical infection where high levels of PrP^{Sc} accumulate in the absence of clinical symptoms are also recognised [74–77]. Prion-infected mice expressing PrP^{C} without a GPI anchor produce infectious prions, accumulate extracellular PrP amyloid plaques, but do not succumb to disease [78]. It has been suggested that PrP^{Sc} needs to interact with cell surface PrP^{C} to exert a neurotoxic effect via aberrant signalling cascades [64,79,80]. An alternative explanation for these experimental observations is that accumulation of high intracellular levels of disease-associated PrP or an intermediate species within neurons themselves is required for cytotoxicity. Other suggested neurotoxic mechanisms relate to altered PrP^{C} trafficking; PrP^{C} can assume two different transmembrane topologies (^{Ctm}PrP-C transmembrane PrP with an extracellular C-terminus and ^{Ntm}PrP-N transmembrane PrP with an extracellular N-terminus) one of which, ^{Ctm}PrP, has been shown to confer severe neurodegeneration in mice with features typical of prion disease [81].

2. Prions and the proteasome

2.1. Ubiquitin proteasome system

The UPS is the primary cellular quality control system in eukaryotic cells for selecting and degrading proteins that are either incomplete, missense, or misfolded, and that could potentially form toxic aggregates [82]. UPS-mediated protein degradation is a highly complex process [83] regulating a number of cellular functions such as cell division, gene expression, differentiation and development, DNA repair, and organelle biogenesis. In this degradation system, a polyubiquitin chain is covalently attached to the substrate, thereby serving as a 'degradation tag', and substrates are degraded by the 26S proteasome into small peptides, with free ubiquitin released for recycling. The 26S is a large, ATP-dependent enzyme complex, resident in both the nucleus and the cytosol of eukaryotic cells. It consists of a 20S proteolytic core and one or two 19S regulatory particles (Fig. 2A). Access to the 20S core is controlled by the six ATPases of the 19S particle [84]. The 20S is a hollow, barrel-shaped protein complex composed of α and β subunits arranged in four stacked rings. These stacked rings surround a central catalytic chamber whereby degradation proceeds without interference from the cytosol. The two α rings form gated channels through which substrates enter and peptides exit the 20S. The proteolytic activity of the 20S resides in its two β rings and consists of three distinct catalytic activities termed β_1 (caspase-like), β_2 (trypsin-like), and β_5 (chymotrypsin-like) and the secondary alcohol of the N-terminal threonine of the active β subunit acts as the nucleophilic species [85]. Following recognition by the 19S, the polyubiquitinated substrate docks onto the 19S particle and is unfolded via the action of six ATPases, which then translocate the substrate and trigger gate opening (Fig. 2B). Docking of the C-termini of the ATPases into intersubunit pockets in the α-ring of the 20S opens the gate in a 'key in a lock' fashion to allow substrate entry [86].

UPS impairment may play a role in neurodegenerative diseases, such as AD, Parkinson's disease (PD), and Huntington's disease (HD), which are all characterised by the accumulation of aggregated misfolded proteins [87]. Proteasomes cannot efficiently degrade aggregated protein [88] and in cell models the functional capacity of the UPS has been shown to be impaired by protein aggregates [89]. At present, its pathogenic role in some of these neurodegenerative diseases is not clear. Polyglutamine pathogenesis has been reported in the absence of significant proteasome impairment in a spinocerebellar ataxia 7 mouse model [90]. In an HD mouse model, there was no detectable impairment of proteasome catalytic function [91]. Recently, however, global changes to the ubiquitin system have been shown in HD [92]; using a mass-spectrometry-based method it was reported

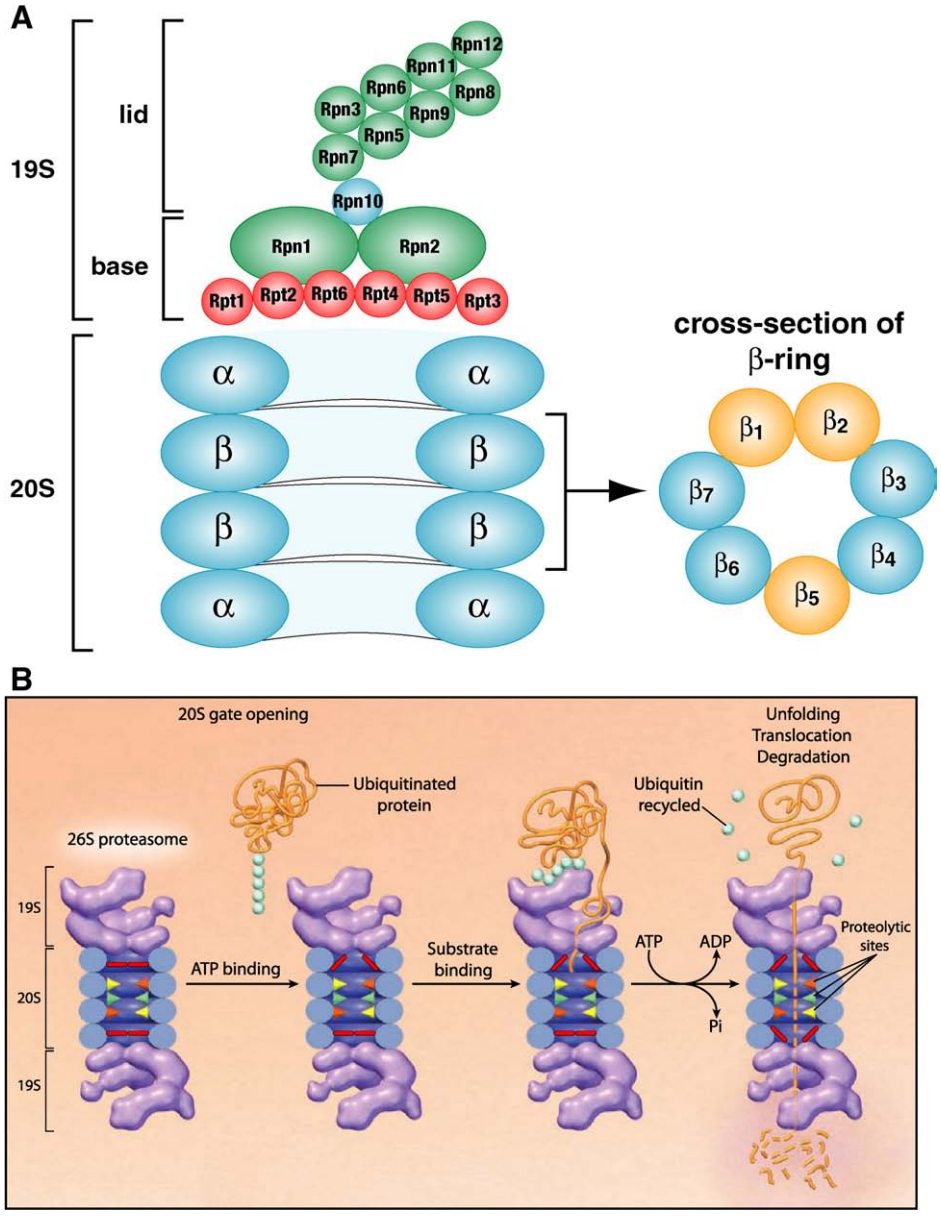

Fig. 2. Ubiquitin proteasome system mediated degradation (A) Schematic of the 26S proteasome in *Saccharomyces cerevisiae*, showing the 20S proteolytic core and one 19S regulatory particle. The 19S is made up of two subcomplexes termed lid and base, the latter consisting of six homologous members of the ATPase family (shown in red) and two larger non-ATPase subunits (Rpn1 and Rpn2). The C-termini of the ATPases dock into intersubunit pockets in the α-rings of the proteasome and open the gate for substrate hydrolysis by the 20S β subunits. Cross section of the β-ring reveals the positions of the caspase-like (β_1), trypsin-like (β_2) and chymotrypsin-like (β_5) subunits (all shown in yellow). Rpn, regulatory particle non-ATPase; Rpt, regulatory particle ATPase. (B) Schematic of UPS-mediated degradation. The 26S degrades proteins in an ATP-dependent manner. The ubiquitinated substrate binds to the 19S where the ubiquitin chain is removed. The 19S ATPases unfold and translocate the substrate through the open gate (shown in red) and into the 20S for proteolysis. ATP denotes adenosine triphosphate, ADP adenosine diphosphate, and Pi inorganic phosphate. B is adapted from Goldberg AL. On Prions, Proteasomes, and Mad Cows. NEJM (2007) 357:1150–1152. Copyright© (2007) *Massachusetts Medical Society. All rights reserved*.

that Lys 48-linked polyubiquitin chains, typically associated with proteasomal targetting, accumulate early in pathogenesis in both transgenic HD mouse brains and human HD patient brain [92].

2.2. The UPS in prion disease

2.2.1. Cytosolic PrP^C and the proteasome

The UPS is involved in ER-associated degradation (ERAD) where ER-resident proteins, in unassembled or misfolded forms, undergo retrograde transport to the cytosol, get ubiquitinated and are normally degraded by the proteasome [93]. Both wild-type and misfolded forms of PrP^C undergo ERAD [94,95]. A pathogenic PrP^C mutant (Y145stop) associated with an inherited prion disease, GSS, is degraded via ERAD [96], whereas another GSS-associated PrP^C mutant (Q217R), remains bound to BiP, an ER chaperone, for an unusually long period of time before proteasomal degradation [97]. More importantly, wild-type PrP^C molecules undergoing ERAD have been shown to accumulate in the cytosol when the proteasome is inhibited [94,95]. Yedidia et al. showed that cells treated with proteasome inhibitors accumulated both detergent-soluble and insoluble PrP^C species, with the latter containing a protease-resistant core and ubiquitin [94]. In a separate study, Ma and Lindquist showed that inhibition of the proteasome in cells led to a significant fraction of endogenous PrP^C accumulation in the cytoplasm [95]. The authors suggest that PrP^C gets to the cytoplasm via retrograde transport. Electron microscopy of mouse brain sections shows that

cytosolic PrP^C is localised in the neurons of the hippocampus, neocortex and thalamus, supporting the fact that cytosolic PrP^C occurs in vivo [98]. UPS impairment may allow the conversion of cytosolic PrP^C to an abnormal, 'PrP^{Sc}-like' form, with partial protease resistance and detergent insolubility [99]. Proteasome inhibition causes the accumulation of PrP^C aggregates in the cytosol of neurons with some PrP^{Sc}-like properties, such as self-sustaining replication and partial PK resistance [99]. It is possible that if the quantity of PrP^C exceeds the degradative capacity of the UPS, then some PrP^C may be able to convert to a protease-resistant pathogenic form as removal of the proteasome inhibitor does not affect the abnormal PrP generation process once it starts [99].

The relationship between UPS inhibition, cytosolic PrP^C accumulation and neurotoxicity in prion disease has been investigated. Studies indicate that cytosolic PrP^C accumulation appears toxic to neurons [66]. It has been shown that cells with higher levels of PrP^C expression are selectively killed by treatment with proteasome inhibitor [100]. In the same study, the authors showed that unglycosylated PrP^C lacking a signal peptide and hence the ability to traffic to the ER, accumulates in the cytosol and is neurotoxic. In agreement with these findings, it has been shown that cytosolic PrP^C accumulates in cells when the function of the PrP^C signal sequence is compromised [101]. More evidence for a toxic role of cytosolic PrP^C comes from a yeast model in which it was shown that during post-translational targeting of PrP^C to the ER, PrP^C is missorted to the cytosol and interferes with cell viability [102]. Additionally, toxicity has been linked to cytosolic PrP^C accumulation, when PrP^C co-aggregates with the anti-apoptotic protein Bcl-2 with toxicity abrogated after over-expression of Hsp70 and Hsp40 [103]. In vivo, mice expressing a PrP^C mutant lacking the N-terminal ER targeting signal (cytoPrP) develop normally, but are severely ataxic, with cerebellar degeneration and gliosis; they also accumulate an insoluble PrP^C form [100].

In spite of the experiments described above, the neurotoxic nature of cytosolic PrP^C in prion pathogenesis is under debate. Cytosolic PrP^C accumulation in human primary neurons treated with proteasome inhibitors has been reported and is not toxic; paradoxically it protects against Bax-mediated cell death [58]. Importantly, there have been observations that neither mutant nor wild-type PrP^C undergo retrograde transport prior to proteasome degradation in various cell models [104,105]. Furthermore, it has been argued that cytosolic PrP^C accumulation may be a result of elevated levels of PrP^C expression from the cytomegalovirus (CMV) promoter used in many of the experiments. Drisaldi et al. show that proteasome inhibition results in the accumulation of unglycosylated cytosolic PrP^C in cells over-expressing PrP^C under the CMV promoter, but not in untransfected cells or Tg mice-derived primary neurons, which express PrP^C from the endogenous promoter [104]. Similarly, Fioriti et al. show that UPS inhibition in transfected cells expressing mouse mutant PrP^C homologues associated with inherited prion diseases causes accumulation of an unglycosylated, aggregated form of PrP^C in transfected cells that is not toxic [105]. In agreement with previous findings [104], the authors show that elevated PrP^C expression leads to cytosolic PrP^C accumulation and suggest this is due to impaired degradation of abortively translocated, signal peptide-bearing molecules synthesised from the CMV promoter.

Moreover, the occurrence of PrP^C retrograde transport has been challenged as antibodies against the signal peptide show that PrP^C accumulates in the cytosol as a result of failed translocation into the ER. In vivo, accumulation of unglycosylated cytosolic PrP^C did not cause any overt phenotype in aged, gene-targeted mice [106]. These lines of evidence suggest that some PrP^C undergoes ERAD, but this proportion is most likely rapidly broken down and is a short lived species [94]. Despite conflicting data, aberrant PrP^C trafficking to the cytosol, which does appear to occur via ERAD [94,95], may play a role in prion pathogenesis but as yet not fully defined.

2.2.2. UPS inhibition and PrP^{Sc} aggresomes

A correlation between elevated levels of ubiquitin protein conjugates and reduced proteasome function in prion-infected mouse brain has been reported [107]. Therefore, proteasome impairment may indeed be important in prion pathogenesis. Large, intracellular, pericentrosomal structures termed aggresomes are thought to be a precise response when cells try to cope with increased levels of misfolded and aggregated proteins, evidenced by the active recruitment of proteasome components and molecular chaperones to these aggregates [108]. PrP^C and PrP^{Sc} aggresome formation has been reported in prion disease models in vitro [109–113]. Wild-type cells treated with cyclosporine A, an immunosuppressant, accumulate proteasome-resistant, 'prion-like' PrP species in aggresomes [109]. When the proteasome is inhibited pathogenic PrP^C mutants have also been reported to accumulate in aggresomes [109]. In vitro, GFP-tagged PrP mutants associated with familial prion disease accumulated in cytosolic, aggresome-like structures after proteasome inhibition, whereas GFP-tagged wild-type PrP^C did not [110]. Moreover, the formation of cytosolic PrP^C aggresomes after transient cytoplasmic PrP^C expression appears to be toxic in both neuronal and non-neuronal cells [111]. Recently, cytoplasmic PrP^C aggresomes were shown to induce cell death in various cell models by modifying the cell stress response via the activation of the RNA-dependent protein kinase and the induction of poly(A)$^+$RNA aggregation [112]. In 2005, we reported that mouse prion-infected neuronal cell lines are more susceptible to cell death after proteasome inhibition [113]. In our cell system we used mild levels of proteasome inhibition, which is suggested to mimic the loss of proteasome activity associated with either the ageing process [114,115] or that may be seen in prion disease in vivo [107,114–116]. In our study, after mild proteasome inhibition, prion-infected cells formed cytosolic aggresomes containing PrP^{Sc}, hsp70, ubiquitin and proteasome subunits [113]. Our work showed that PrP^{Sc} aggresome formation was temporally associated with caspase 3 and 8 activation, and subsequent apoptosis in prion-infected cells [113]. In our cell system, PrP^{Sc} aggresome formation and subsequent cell death were abrogated after treatment with microtubule inhibitors which prevented aggresome formation [113]. In our study, we found evidence for PrP^{Sc} aggresome-like structures in prion-infected mouse brain [113]. Granular deposits of disease-related PrP have been previously reported in neuronal perikarya from postmortem sporadic CJD cases, suggesting intra-neuronal prion aggregates may play a role in disease pathogenesis [117].

At present, PrP^{Sc} trafficking is poorly defined due to the lack of PrP^{Sc}-specific antibodies and hence the way PrP^{Sc} may enter the cytoplasm to form aggresomes has not been established [42]. Possibilities include retrotranslocation from the ER [94,95] or via endolysosomal membrane destabilisation and leakage into the cytosol, as described for $A\beta_{1-42}$ [118]. UPS impairment due to the aging process or during prion infection in vivo [116], may allow for PrP^{Sc} accumulation into toxic aggresomes. A key question is whether this accumulation is accompanied by concomitant buildup of a distinct neurotoxic intermediate oligomeric species.

2.3. PrP oligomers and the UPS

There is growing consensus that prefibrillar soluble protein aggregates, rather than insoluble fibrils, may in fact be the toxic species in neurodegenerative disease [119]. Nonetheless, this theory is currently limited due to the lack of a precise physical definition for these prefibrillar soluble protein aggregates, or 'oligomers'. The molecular mechanisms of prion protein misfolding, and the role of intermediate states when the prion protein transits from an α-helical to a β-sheet-rich structure has not yet been possible to define. Structural studies of recombinant PrP molecules demonstrate that the protease-resistant core can distinctly fold not only into amyloid fibrils, but also into β-sheet-rich oligomers [120–122]. Although the size of such β-oligomers

remains poorly defined, there have been some attempts to characterise their structure [120,123–125]. The proposed oligomeric precursor of PrP^{Sc} could have similar properties to prefibrillar species seen in AD [126]. Prion oligomers have been implicated to play a role in toxicity and infectivity in prion disease [127,128].

In our most recent work, we found evidence that misfolded PrP triggers a potential neurotoxic mechanism that may explain why extracellular PrP^{Sc} itself is not toxic [116]. We demonstrated a significant loss of chymotrypsin-like (β_5) and caspase-like (β_1) proteolytic activity in prion-infected cells and prion-infected mouse brain, with a lesser effect on trypsin-like (β_2) activity. We also presented evidence for inhibition of the catalytic β-subunits of the 26S proteasome in vitro by recombinant β-sheet-rich forms of PrP (β-PrP) [129] and semi-purified PrP^{Sc} from RML prion-infected mouse brain. This inhibition occurred at near one to one stoichiometry inferring that the inhibitory species is highly potent and binds with tight affinity. The inhibitory effect on the catalytic sites was limited to only certain aggregated β-sheet-rich conformational isoforms of PrP [116]. Pre-incubating our β-PrP and PrP^{Sc} species with an antibody raised against aggregation intermediates [130] abrogated the inhibitory effect on proteolysis, suggestive of an oligomeric inhibitory species [116]. We also used cell-based GFP-tagged proteasome reporter substrates, and found that prion-infected mouse neuroblastoma cells accumulated the GFP reporter indicative of functional UPS impairment in live cells (Fig. 3A). Using a transgenic mouse model that allows the functional status of the UPS to be

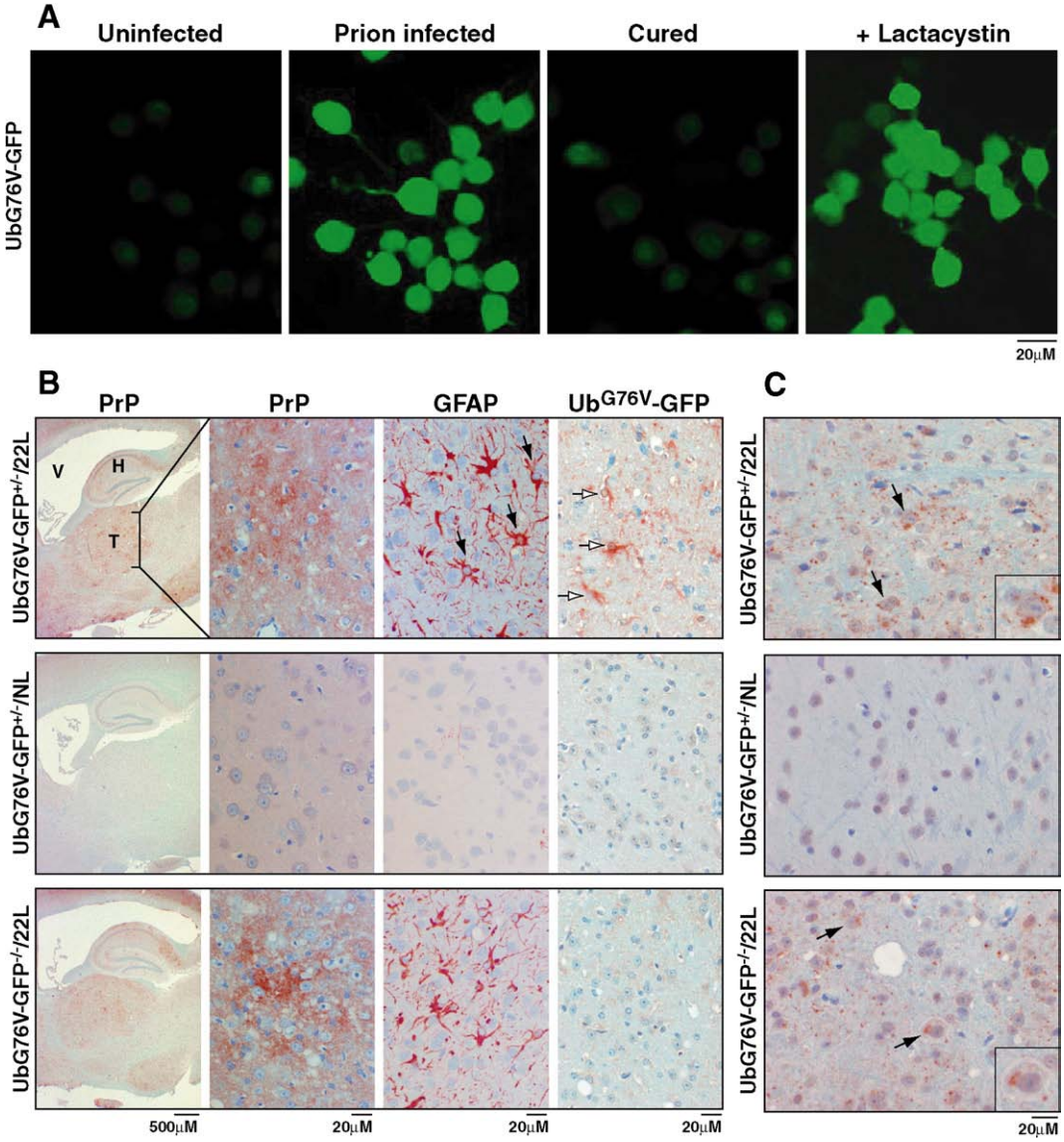

Fig. 3. Disease-associated prion protein oligomers inhibit the 26S proteasome (A) The GFP-proteasome reporter accumulates only in prion-infected GFP-reporter expressing N2aPK-1 neuroblastoma (UbG76V-GFP) cells or proteasome inhibitor-treated (50 μM lactacystin) N2aPK-1 cells indicating functional proteasome impairment in these cells. Cured denotes cells treated with anti-PrP antibody. (B) Immunohistochemical studies of GFP-proteasome reporter transgenic mice (UbG76V-$GFP^{+/-}$) inoculated intracerebrally with a mouse scrapie prion strain (22L) (top) or normal brain homogenate (NL) (middle), and nontransgenic littermates inoculated with 22L prions (bottom). Left panels show low-power views of saggital sections through the hippocampus (H), third ventricle (V), and thalamus (T) stained for PrP. Other panels show high-power views of the thalamus stained for PrP, GFAP, and Ub^{G76V}-GFP. The 22L prion-inoculated mice exhibited primarily diffuse PrP staining and extensive gliosis (black arrows point to hypertrophic astrocytes). Collections of Ub^{G76V}-GFP+ cells were seen in the 22L inoculated transgenic mice (open arrows), but not in mock-infected Ub^{G76V}-GFP transgenic mice or 22L prion-infected nontransgenic littermates (middle and bottom panels). (C) Ubiquitin staining in sections of thalamus from UbG76V-$GFP^{+/-}$ mice inoculated with 22L prions or normal brain homogenate. Ubiquitin granular deposits are seen scattered throughout the sections of the 22L prion-infected brain sections (top and bottom panels) but rarely observed in the control (middle panel). Arrows point to large neuron-like cells containing ubiquitin deposits that exhibit a perinuclear localization. The bottom panel showing a section from a UbG76V-$GFP^{-/-}$ nontransgenic littermate inoculated with 22L prions demonstrates that the expression of the transgene appeared to have no effect on the deposition of ubiquitinated proteins. From *Molecular Cell, 26, Kristiansen et al., Disease-associated prion protein oligomers inhibit the 26S proteasome, p175–188 Copyright© (2007) with permission from Elsevier.*

directly monitored [131], we found that prion infection also caused specific inhibition of the UPS in these GFP-reporter transgenic mice in vivo. We demonstrated that GFP reporter accumulation and intracellular cytosolic granular ubiquitinated deposits occurred in prion-infected mice only, particularly in the regions of prion-induced neuropathology, indicative of impairment of the UPS (Fig. 3B, C) [116]. Interestingly, the demonstration of functional proteasome impairment in prion infection in vivo may explain earlier reports of similar ubiquitin–protein conjugates within neurons in prion-infected mouse brain seen early in the course of the disease [132]. Using a number of different biochemical approaches we were able to demonstrate that oligomeric PrP species exerted their inhibitory effects on the 20S proteasome itself [116]. Our data can be explained mechanistically by 'oligomeric' PrP species having a direct inhibitory effect on the β_1 and β_5 proteolytic active sites of the 20S core, but this is unlikely due to steric hindrance as the diameter of the gated channel is only ~2 nm [133]. The other possible explanation is that prions inhibit gate opening of the 20S. This second possibility is supported by studies of gate opening where addition of hydrophobic peptides, use of the PA28 activator, or gate deletion (which all accelerate substrate entry) stimulate cleavage at the chymotrypsin-like (β_5) and caspase-like (β_1) sites by enhancing their V_{max}, but do not stimulate the slower cleavage at the trypsin-like site (β_2) [134]. Therefore, an inhibitory effect of prions on 20S gate opening would have a more severe inhibitory effect on the chymotrypsin-like and caspase-like activities rather than the trypsin-like site—an effect seen in our published data [116] and further supported by our current work (Deriziotis et al., unpublished data). Other key questions that arise from this work are the route whereby misfolded forms of disease-associated PrP enter the cytosol, and the role of other degradation pathways, such as autophagy, in prion pathogenesis.

2.4. Autophagy and other pathways in prion protein clearance

In neurodegenerative disease, abnormal proteins with aberrant conformations often aggregate into inclusions. Autophagy, the bulk degradation of cytoplasmic proteins and organelles, may be the principal clearance mechanism by which cells degrade diffuse or oligomeric protein [135]. Induction of autophagy enhances expanded polyQ and α-synuclein clearance in cell models [136], and has been shown to attenuate the toxicity of mutant huntingtin fragments, and both mutant and wild-type tau [137,138]. Autophagy is not well characterised in prion disease. Evidence of autophagy in prion disease is limited to EM showing autophagosomes in experimental models of scrapie, and in human CJD and GSS [139], suggesting it may play a role in intracellular prion protein clearance.

Understanding prion protein clearance mechanisms in prion disease is clearly important in understanding aspects of pathogenesis. Apart from autophagy, which may play a role in clearance, extracellular prion protein clearance by microglia has recently been demonstrated [140]. Glial cells, such as microglia, do not replicate prions [141], even though microglia activation has been documented in prion disease [142]. Interestingly, however, when prion organotypic slice culture assays (POSCA) are depleted of microglia they a) accumulate high levels of PrP^{Sc}, b) have 15 fold higher prion titers and c) are more susceptible to infection when compared to microglia-containing slices [140]. Microglia may indeed have an important role in controlling prion infection by clearing misfolded prions via phagocytosis.

3. Concluding remarks

Whilst prion infection causes widespread neuronal loss in the brain, the molecular basis of prion neurotoxicity is not understood. Neurodegeneration cannot be explained by a loss of functional PrP^{C}, as its depletion does not trigger any gross pathology. The alternative explanation, that PrP^{Sc} itself is directly neurotoxic, does not adequately explain many observations where neurons can be in close proximity to PrP^{Sc} deposits without suffering deleterious effects.

The pathogenesis of prion disease is likely to be multifactorial, but the potent inhibition of the proteasome by pathogenic PrP may result in neuronal perturbation and contribute to the widespread neuronal loss. It is not yet clear whether proteasome impairment is a primary or secondary event (due to upstream cellular dysfunction) in prion disease pathophysiology, and work is ongoing to try and clarify this. Proteasome inhibition by prions also explains how cytosolic aggregated forms of PrP^{C} may accumulate in prion disease, with UPS inhibition leading to the cytosolic accumulation of PrP^{C} destined for ER-associated degradation. Impairment of the UPS leads to cellular dysfunction and apoptosis through many different mechanisms, as the UPS is also involved in transcriptional regulation, cell cycle control, and control of apoptosis [82]. Given the significant role that has emerged for the UPS in protein-misfolding disorders combined with the age-dependent decrease in UPS activity, designing drugs that improve neuronal UPS function may offer a successful intervention to slow or prevent neurodegenerative diseases in which toxic proteins are misfolded.

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