Acta Biochim Biophys Sin 2013, 45: 477–484 | © The Author 2013. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmt020.
Advance Access Publication 28 February 2013

Review

Interaction between misfolded PrP and the ubiquitin-proteasome system in prion-mediated neurodegeneration

Zhu Lin, Deming Zhao, and Lifeng Yang*

State Key Laboratories for Agrobiotechnology, National Animal Transmissible Spongiform Encephalopathy Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
*Correspondence address. Tel/Fax: +86-10-62732987; E-mail: yanglf@cau.edu.cn

Prion diseases are associated with the conformational conversion of cellular prion protein (PrP^{C}) to pathological β-sheet isoforms (PrP^{Sc}), which is the infectious agent beyond comprehension. Increasing evidence indicated that an unknown toxic gain of function of PrP^{sc} underlies neuronal death. Conversely, strong evidence indicated that cellular prion protein might be directly cytotoxic by mediating neurotoxic signaling of β-sheet-rich conformers independent of prion replication. Furthermore, the common properties of β-sheet-rich isoform such as PrP^{Sc} and β amyloid protein become the lynchpin that interprets the general pathological mechanism of protein misfolding diseases. Dysfunction of the ubiquitin-proteasome system (UPS) has been implicated in various protein misfolding diseases. However, the mechanisms of this impairment remain unknown in many cases. In prion disease, prion-infected mouse brains have increased levels of ubiquitin conjugates, which correlate with decreased proteasome function. Both PrP^{C} and PrP^{Sc} accumulate in cells after proteasome inhibition, which leads to increased cell death. A direct interaction between 20S core particle and PrP isoforms was demonstrated. Here we review the ability of misfolded PrP and UPS to affect each other, which might contribute to the pathological features of prion-mediated neurodegeneration.

Keywords prion disease; ubiquitin-proteasome system; proteasome; misfolded PrP

Received: October 21, 2012 Accepted: December 10, 2012

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), develop as central nervous system disease after long incubation periods, and many of which may arise following the consumption of infected materials. 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 biological character of the transmissible agent.

The infectious agent is a misfolded and partially proteinase K (PK)-resistant form of prion protein (scrapie associated PrP-PrP^{Sc}), which directs normal host prion protein to accumulate in the misfolded form. 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. The appearance of PrP^{Sc} is the initiation of prion diseases, and the misfolded PrP has a nature of conversion and a trend of aggregation.

The ubiquitin-proteasome system (UPS) is the primary cellular quality control system in eukaryotic cells for selecting and degrading proteins that are either incomplete, missense, or misfolded, and could potentially form toxic aggregates. UPS is responsible for the degradation of >80% of normal and abnormal intracellular proteins, including many cellular polymers and misfolded proteins [1]. Aberrations in this system have been implicated in the etiology of neurodevelopmental and neurodegenerative diseases, such as mutations in ubiquitin carboxy-terminal hydrolase (UCH-L1), α-synuclein (PARK1), and Parkin (PARK2) [2]. Kristiansen *et al.* [3] reported that mouse prion-infected neuronal cell lines are more susceptible to cell death after proteasome inhibition. *Hectd2* gene that encodes a ubiquitin ligase was identified as a gene influencing incubation time for prion disease in mice [4]. Hence, we suggest a linkage between the UPS and prion diseases, which may be important in the neurotoxic mechanism of prion diseases.

Prion Disease

The prototypic prion disease is scrapie, a common disease affecting sheep and goats. Human prion diseases include

Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia, and kuru. Mammalian prion diseases include transmissible mink encephalopathy, chronic wasting disease of deer and elk, and bovine spongiform encephalopathy in cattle.

The term 'prion' was first proposed by Stanley Prusiner to distinguish the infectious particle in prion disease as a pathogen from bacteria and viruses, and defined as a 'small, proteinaceous infectious particle that resists inactivation by procedures that modify nucleic acids' [5]. The research for scrapie came to be the milestone of the discovery of prion. Stanley Prusiner found that the pathogen of scrapie was insusceptible to any kinds of nuclease. Furthermore, while nucleic acid is vulnerable to ultraviolet radiation and ultrasonic wave, scrapie pathogen has the resistance to various factors of physical inactivation. On the other hand, treatments impacting proteins can weaken or inactivate the infectivity of the pathogen. Progressive enrichment of scrapie-infected hamster brain homogenates for infectivity led to the isolation of a protease-resistant polypeptide which was later identified as part of the prion protein (PrP) [6].

Griffith [7] first introduced the possibility that the material responsible for disease transmission might be a protein that had the surprising ability to replicate in the body. This launched the so-called 'protein-only' hypothesis of TSE propagation, subsequently moved forward by Stanley Prusiner's group [5]. In the 'protein-only' hypothesis, prion diseases are thought to be resulted from the conformational change of a normal isoform of a prion protein (PrP^{C}) to a protease-resistant, pathogenic form called PrP^{Sc} which recruits endogenous PrP^{C} in order to replicate, and forms the quantitative to qualitative changes that damage the nervous tissue. Although they share the same amino acid sequence, PrP^{C} is mainly α-helical, whereas PrP^{Sc} is β-sheet rich. In addition, PrP^{Sc} is less soluble in detergents and more resistant to proteases. With the amplification of infectious prions *in vitro* [8], and their recent generation by protein misfolding cyclic amplification from purified recombinant prion protein with the sole addition of RNA and lipids [9], the evidence for the protein-only prion model is now overwhelming.

Despite extensive research, the exact nature of the infectious agent is still unknown. This mystery is highlighted by the lack of correlation between infectivity and PrP^{Sc} levels [10–12] as well as by the existence of PK-sensitive forms of PrP^{Sc} [13–19]. The facts that minimal infectivity was associated with protein-only inoculations and highly infectious prion could be generated in the presence of lipids suggest that accessory co-factor molecules may be essential for prion infectivity in mammals [20–22]. And such a co-factor might increase the biological stability of prions, thereby reducing their *in vivo* clearance. Although it is clear that PrP^{Sc} is the main informational molecule in the infectious agent, it remains possible that co-factor molecules are required [21].

The Ubiquitin-Proteasome System

UPS is consisted of a ubiquitin binding system and a 26S proteasome. The ubiquitin binding system contains a series of enzymes: ubiquitin-activating enzyme, E1; ubiquitin-conjugating enzyme, E2; ubiquitin-protein ligase, E3; and deubiquitinating enzymes. The proteasome is a multicatalytic protease that degrades polyubiquitinated proteins to short peptides. It is composed of two subcomplexes: a 20S core particle (CP) which carries the catalytic activity and a regulatory 19S regulatory particle (RP) which is involved in the recognition of the substrate [23].

The degradation of a protein *via* the ubiquitin-proteasome pathway involves two discrete and successive steps [24]: (i) tagging of the substrate by covalent attachment of multiple ubiquitin molecules to synthesize the polyubiquitin chain proteolytic signal and (ii) degradation of the tagged protein by the 26S proteasome complex with release of free and reusable ubiquitin catalyzed by ubiquitin-recycling enzymes also known as deubiquitinating enzymes (DUBs) (**Fig. 1**). Conjugation of ubiquitin, a highly evolutionarily conserved 76 amino acid residue polypeptide, to the protein substrate proceeds *via* a three-

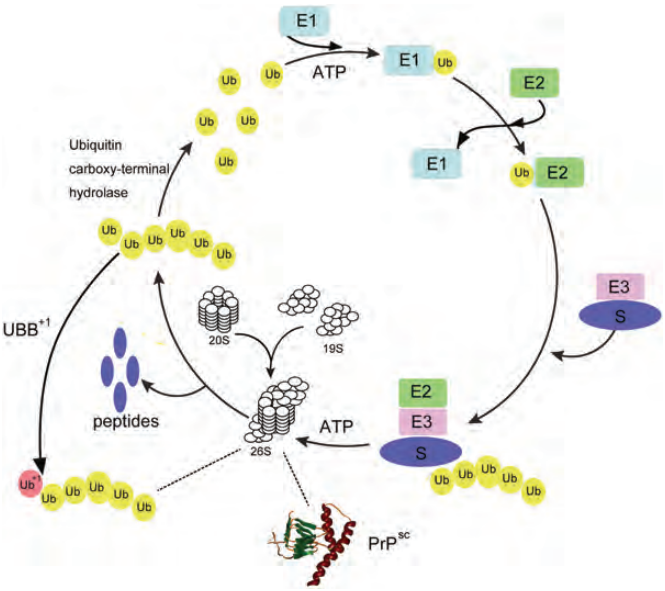

Figure 1 The ubiquitin-proteasome pathway of degradation Substrate proteins are mono- or multiubiquitylated by the actions of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligase (E3) enzymes, a modification reversed by DUB enzymes. The dotted line indicates: (i) ubiquitin^{+1} 'capped' unanchored multi-ubiquitin chains impairs 26S proteasome function; (ii) protein aggregates in the pathological state also directly inhibit 20S and 26S function, further contributing to inclusion formation.

step cascade mechanism. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), all of which are available and indeed used *in vivo* for chain extension [25]. The significance of complex ubiquitylation patterns is only partially understood: K48 chains (which are the most abundant) lead to degradation of the substrate by the 26S proteasome, whereas monoubiquitylation and K63 chains do not specify degradation but have other biological functions, including marking proteins for endocytosis [26]. Initially, E1 activates ubiquitin in an ATP-requiring reaction to generate a high-energy thiol ester intermediate, E1-S∼ubiquitin. One of several E2 enzymes then transfers the activated ubiquitin from E1 *via* an additional high-energy thiol ester intermediate, E2-S∼ubiquitin, to the substrate that is specifically bound to E3. E3s catalyze the last step in the conjugation process, namely the covalent attachment of ubiquitin to the substrate. The 20S CP is a barrel-shaped structure composed of four stacked rings: two identical outer α rings and two identical inner β rings. The eukaryotic α and β rings are each composed of seven distinct subunits, giving the 20S complex the general structure of α1-7β1-7β1-7α1-7. The catalytic sites are localized to some of the β subunits. The 19S regulatory complex binds flexibly to the 20S catalytic core and consists of six ATPases, forming a ring at the entrance of the core and exerting chaperone-like activity [27]. In addition to recognizing ubiquitinated proteins and other potential substrates of the proteasome, a second function of the 19S RP is to open an orifice in the α ring that will allow the entry of the substrate into the proteolytic chamber. Following degradation of the substrate, short peptides derived from the substrate are released along with reusable ubiquitin. These peptides are further degraded to amino acids by cytosolic amino and carboxypeptidases.

Prion and Ubiquitin-Proteasome System

Role of misfolded PrP in the impairment of UPS

It has been reported that prion-infected mouse brains have increased levels of ubiquitin conjugates, which correlates with decreased proteasome function [28]. A number of researches have also been done to explain how misfolded PrP interact with UPS [29−31]. Another study revealed an intracellular mechanism whereby toxic prion oligomers impair the proteasome *in vivo* by inhibiting the catalytic β subunits of the 20S proteasome [29]. It was found that prion infection caused an increase in the steady-state levels of the reporter, indicative of functional impairment of the UPS. Modification of β subunit activities (indicating a loss of activity) was clearly seen in prion-infected cells. The establishment of a transgenic mouse model allowed the functional status of the UPS to be monitored, and the data suggested that prion disease *in vivo* was associated with UPS dysfunction and supported the *in vitro* studies, indicating that proteasome function was directly compromised by the presence of PrP^{Sc} or its synthetic mimetic β-PrP. Deriziotis *et al.* [32] reported that aggregated β-PrP has a high affinity for the 20S particle, and reduced the 20S proteasome basal peptidase activity by stabilizing the closed conformation of the substrate entry channel, which could account for the inhibition of 26S proteasomal function. A recent study suggested a mechanism of proteasome impairment by the direct interaction of β-sheet-rich PrP to reduce gate opening and inhibit substrate entry [33]. This novel mechanism may provide a model for how misfolded, disease-associated proteins might interact with the proteasome and disrupt its function.

The impairment of UPS plays a role in prion infection

The UPS is involved in endoplasmic reticulum (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 [34]. Lindquist and colleagues [35] first reported that PrP retrogradely transported out of the ER produced the accumulation of PrP^{C} aggregates with some PrP^{Sc}-like properties (such as protease resistance and detergent insolubility) in the cytosol of neurons with proteasome inhibition. And transient proteasome inhibition is sufficient to initiate sustained PrP conversion; even removal of the proteasome inhibitor does not affect the abnormal PrP generation process once it starts. These observations might explain the origin of PrP^{Sc}. Both wild-type and misfolded forms of PrP undergo ERAD [36,37]. A pathogenic PrP^{C} mutant (Y145stop) associated with an inherited prion disease, GSS, is degraded *via* ERAD [38], 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 [39]. More importantly, wild-type PrP^{C} molecules undergoing ERAD have been shown to accumulate in the cytosol when the proteasome is inhibited [36,37]. Cells treated with proteasome inhibitors showed to accumulate both detergent-soluble and -insoluble PrP^{C} species, with the latter containing a protease-resistant core and ubiquitin [36]. A separate study showed that inhibition of the proteasome in cells led to a significant fraction of endogenous PrP^{C} accumulation in the cytoplasm [37]. With aging, cellular repair mechanisms are characterized by a decrease of protein repair and an increased formation of aggresomes [40]. Therefore, UPS impairment, due to the aging process or during prion infection *in vivo*, may allow for PrP accumulation into toxic aggresomes. The impairment of the UPS may play an important role in prion diseases characterized by accumulation of misfolded proteins, but the biochemical mechanisms underlying this dysfunction remain unclear. Increasing evidence suggested that soluble micro-aggregates of misfolded proteins, rather than

larger protein inclusions, are toxic to neurons in neurodegenerative diseases [41]. Thus, it is possible that such aggregates eventually overwhelm the UPS, causing a functional impairment and further pathogenic transformation.

The relationship among UPS inhibition, cytosolic PrP accumulation, and neurotoxicity in prion disease has been widely investigated. Studies indicated that cytosolic PrP accumulation appears to be toxic to neurons [42]. Straight and inducible expression of cytosolic PrP in transgenic mice leads to neurodegeneration in different neuronal populations [43,44]. Cells with higher levels of cytosolic PrP expression are selectively killed by treatment with proteasome inhibitor [44]. More evidence for a toxic role of cytosolic PrP 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 [45]. In addition, Rambold *et al.* [46] used a mutant that contains the N-terminal ER-targeting signal but lacks the C-terminal glycosylation phosphatidylinositol (GPI) signal sequence (PrPΔGPI, which can spontaneously adopt a misfolded conformation) to analyze the subcellular localization of the different PrP constructs. The results showed that only in the cytosol the accumulation of misfolded PrP induced apoptosis. Apoptotic cell death was also induced by two pathogenic mutants of PrP, which are partially localized in the cytosol. Experiments described above proved the neurotoxic nature of cytosolic PrP in prion pathogenesis. Strong evidence indicated that cellular prion protein might be directly cytotoxic by mediating neurotoxic signaling of β-sheet-rich conformers independent of prion replication [47]. Paradoxically, cytosolic PrP accumulation in human primary neurons treated with proteasome inhibitors has been reported: instead of causing cell toxicity, it protects against Bax-mediated cell death [48]. Despite much conflicting data, aberrant PrP^{C} trafficking to the cytosol, which does appear to occur *via* ERAD [36,37], may play a role in prion pathogenesis. UPS plays an important role in the dispose of misfolded PrP in the cytosol of neurons. Thus, the speed of cytosolic PrP accumulation beyond the UPS capacity or the inhibition of UPS may account for the aggregation of cytosolic PrP and its toxicity collection. Actually, the 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 [49].

UPS functionality increases in prion-associated disease

Oxidative stress is another important factor that has been implicated in the pathogenesis of a number of neurodegenerative diseases, which can account for the activation of the proteasome complex relatively less reported in neurodegenerative diseases such as prion disease, Alzheimer's diseases (AD), and Parkinson's diseases (PD). A proteomic analysis reported that ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) found to be enriched in AD [50]. Amici *et al.* [51] reported an increase of proteasome functionality in prion-associated disease and a higher incidence of protein oxidation in both aged and scrapie-infected subjects. Previous publications have reported that oxidized proteins are preferentially degraded by the 20S proteasome [52,53] and that mild oxidative conditions may regulate and enhance proteasome-mediated degradation [54–57]. Thus, the activation of the proteasome complex may be considered as a way of counteracting the effects of oxidative stress. Another proteomic analysis revealed that the UCH-L1 is a major target of oxidative damage in AD and PD brains, suggesting a direct link between oxidative damage and the neuronal ubiquitination/de-ubiquitination machinery [58]. One of the major consequences of aberrant UCH-L1 activity is an impaired proteasome proteolytic system, which will lead to accumulation of damaged proteins and formation of protein aggregates. Therefore, the confrontation process between UPS and oxidative damage or misfolded PrP may show us a peak shape function of the activity of UPS. If the counteracting capacity cannot balance the severe oxidative stress, UPS would suffer the oxidative damage and cause protein-misfolding disorders.

Proteins involved in perturbed UPS potentially serve as disease biomarkers

To control and prevent prion diseases, an early diagnosis is urgently needed. Further analysis of proteins involved in perturbed UPS may help elucidate mechanisms behind misfolded PrP pathogenesis and provide new sources of biomarkers.

Ubiquitin-B (UBB) and UBB-like proteins play a central role in the UPS. In AD brain areas where cell death has been reported (i.e. the hippocampal region CA1 and the subiculum), the expression of mutant ubiquitin that indicated the inhibition of proteasome might reach a critical level [59]. UBB^{+1} (a frameshift mutant form of ubiquitin) has been observed in the hallmarks of AD, as well as other tauopathies and in polyglutamine diseases (e.g. Huntington's disease). It has been shown *in vitro* and *in vivo* that UBB^{+1} inhibits the proteasome and gives rise to downstream effects (e.g. a behavioral phenotype; impaired contextual memory) [60] (**Fig. 1**). UBB^{+1}, as a result of genomic mutations due to dinucleotide deletions, may lead to an accumulation in hallmark structures neurofibrillary tangles (NFTs), senile plaques, and neuropil threads [61]. A neuropathological analysis revealed the deposition of PK-resistant prion protein, abnormal accumulation of mitochondria in the vicinity of PrP deposits, and expression of mutant ubiquitin (UBB^{+1}) in NFTs and dystrophic neurites [62].

One of the most common types of familial PD is caused by mutations in *PARK2*, which encodes the E3 ubiquitin ligase parkin. Mutations in *PARK2* cause early disease onset, with the loss of dopaminergic neurons in the substantia nigra in the general absence of Lewy bodies [63]. The first E3 ligase to be implicated in long-term potentiation was *UBE3A* (also known as E6-AP) [64], the mutation of which causes Angelman syndrome in humans, a disease that is associated with defects in neuronal development. Furthermore, *UBE3A* is localized to dendrites and spines, and its absence leads to reduced spine density and length [65]. In addition to *PARK2* and *UBE3A*, an increasing number of E3 genes are linked to neurogenetic disorders, including *UBR1* (Johanson–Blizzard syndrome) [66], *NHLRC1* (Lafora's disease) [67], and *CUL4B* [68], *BRWD3* [69] and *HUWE1* [70].

About 100 DUBs have been identified in the human genome, and most of them belong to two major families: ubiquitin-specific processing proteases (UBPs) and ubiquitin C-terminal hydrolases (UCHs) [71]. UBPs are mainly responsible for cleaving ubiquitin–substrate and ubiquitin–ubiquitin isopeptide bonds, reversing protein ubiquitylation. UCHs, on the other hand, remove small adducts from the ubiquitin to maintain the monomeric pool. UCH-L1 plays a crucial role for the clearance of abnormal proteins in UPS. It is one of the most abundant proteins, making 12% of soluble proteins, and is exclusively localized in neurons [72]. It has multiple functions: (i) it is a deubiquitinating enzyme; (ii) it is a ligase; and (iii) it stabilizes monoubiquinated proteins. Down-regulation of UCH-L1 occurs in PD and AD. Its concentration is inversely proportional to the number of tangles [51]. However, liquid chromatography-tandem mass spectrometry analysis identified that proteins involved in the pathogenesis of AD and UCHL1 was found to be enriched [46]. A20 (also called TNFaip3) [73,74], the N-terminal domain of which is a deubiquitinating ligase, regulates the expression of proinflammatory genes in response to various stimuli. Most types of cells do not constitutively express A20, but rapidly up-regulate *A20* mRNA expression after stimulation of nuclear factor-kappaB (NF-κB), which links A20 to the negative feedback regulation of NF-κB activation. The results suggested that paracaspase-mediated A20 cleavage in T lymphocytes is necessary for a proper NF-κB activation [75].

BAG-1 is a binding partner of Hsp70. When BAG-1 is overexpressed, it plays an inhibitory role in the degradation of Tau and consequently results in an accumulation of Tau in neurons and contributes to tangle formation [76]. The cellular PrP binds to the C-terminus of Bcl-2 [77], and PrP may disrupt the chains of Bcl-2 molecules at the homomeric association site in the transmembrane region [78]. Additionally, toxicity has been linked to cytosolic PrP accumulation, when PrP^{C} co-aggregates with the anti-apoptotic protein Bcl-2 with toxicity abrogated after overexpression of Hsp70 and Hsp40 [46]. Another protein, phosphocatenin, when accumulating in the form of aggresomes, may contribute to a proteasome dysfunction [79].

Sporadic forms of AD and PD diseases are the most frequent forms, and together with prion disease, they belong to the so-called protein misfolding disorders as they share a common feature in the accumulation of misfolded protein aggregates. In addition, those diseases cause neurodegenerative effects. Therefore, the UPS in those diseases and the possible consequences due to the accumulation of aberrant proteins may accordingly share some common features. Proteins of UPS involved in AD, PD may have a key position in further researches of identification of the components of this system in prion diseases.

Conclusion

The mechanism of cell death in prion diseases is unknown but is associated with the production of a misfolded conformer of the prion protein. The deficiency of functional PrP^{C} cannot make clear the gross pathology of neurodegenerative disease. Another explanation that PrP^{Sc} itself is neurotoxic does not adequately explain many observations: in transgenic mice where neuronal PrP^{C} is deleted during the course of infection, but extraneuronal replication of PrP^{Sc} continues [80], neurotoxicity does not occur despite extensive PrP^{Sc} accumulation, as well as in mice in which the GPI-anchor is removed, detaching PrP from the cell surface but allowing replication to occur away from the cell bodies and processes [81]. Misfolded PrP accumulation in the endolysosomal system causes dysfunction of the degradative pathway, resulting indirectly in an increased burden on the UPS. The degradative capacity of the UPS is also known to decline during the aging process [82], and combined with the effects of neuronal stress in the brain, this will further incapacitate UPS function, resulting in neuronal dysfunction [83]. Recent studies have identified a possible pathway for prion clearance that involves milk fat globule-EGF factor 8 protein (MFGE8)-mediated phagocytosis of prions by microglia and a co-factor that could reduce the affinity of PrP^{Sc} for MFGE8 [84]. It reminds us of the existence of co-factors acting on UPS, thereby possibly reducing the infectious agent clearance *in vivo*. It is not yet clear whether the UPS impairment is a primary or a secondary event in prion disease pathophysiology, and work is still ongoing to clarify this ambiguity. Whatever the pathogenic factor might be, misfolded, disease-associated PrP, accessory co-factor molecules or oxidative damage, there can always be a disruption of UPS function. Thus, misfolded PrP accumulation beyond the UPS capacity or UPS impairment in a variety of ways may account for the aggregation of misfolded PrP and its toxicity collection.

It is possible that a mutual promotion exists between the impairment of the UPS and the accumulation of misfolded PrP in the neural cytosol (**Fig. 1**).

The pathogenesis of prion disease is likely to be multifactorial, but the potent inhibition of the proteasome by pathogenic PrP is likely to result in neuronal perturbation and contribute to the widespread neuronal loss. The key feature of prion disease, autocatalytic conversion of PrP^{C} to PrP^{Sc}, may form a positive feedback loop by inhibiting the UPS. 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 strategy to slow or prevent neurodegenerative diseases in which misfolded proteins are toxic. Therapeutic use of degradation pathways in prion disease to overcome a dysfunction in protein homeostasis may turn a 'curse' into a 'blessing'. UPS-targeted therapeutic interventions will provide new perspectives for our combat against neurodegenerative disorders over the years.

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