

## Mechanisms of prion-induced neurodegeneration

PAULA SAÁ<sup>1\*</sup>, DAVID A. HARRIS<sup>2</sup>, LARISA CERVENAKOVA<sup>1</sup>

<sup>1</sup>Scientific Affairs, Holland Laboratory, American Red Cross, Rockville, MD, USA, and <sup>2</sup>Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal neurodegenerative disorders characterised by long incubation period, short clinical duration, and transmissibility to susceptible species. Neuronal loss, spongiform changes, gliosis and the accumulation in the brain of the misfolded version of a membrane-bound cellular prion protein (PrP<sup>C</sup>), termed PrP<sup>TSE</sup>, are diagnostic markers of these diseases. Compelling evidence links protein misfolding and its accumulation with neurodegenerative changes. Accordingly, several mechanisms of prion-mediated neurotoxicity have been proposed. In this paper, we provide an overview of the recent knowledge on the mechanisms of neuropathogenesis, the neurotoxic PrP species and the possible therapeutic approaches to treat these devastating disorders.

### Animal and human prion diseases

The term prion was originally coined by S.B. Prusiner to denote a small proteinaceous infectious particle, which is resistant to most procedures that inactivate nucleic acids (Ref. 1). Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders affecting humans and animals (Fig. 1). Human TSEs are often categorised with other protein misfolding neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, fronto-temporal dementia and amyotrophic lateral sclerosis (Ref. 2). These diseases share a common mechanism that involves a conformational change in the structure of the disease-implicated protein, leading to self-replicating propagation and subsequent pathological changes within the central nervous system (CNS). However, only TSEs are known to cause infections of epidemic proportions in humans (kuru) and animals [bovine spongiform encephalopathy (BSE)], and be endemically present in domestic (scrapie) and wild animals [chronic wasting disease (CWD)] (Fig. 1).

Human TSEs can be subdivided into three aetiological groups: sporadic, genetic and environmentally acquired (i.e. infectious). Sporadic Creutzfeldt–Jakob disease (sCJD) is the most common form, occurring without any obvious cause with a frequency of 1 case per million people per year worldwide (Ref. 3). Sporadic fatal insomnia (sFI) is a very rare disease described in only approximately 2 dozen cases so far (Refs 3, 4). The spectrum has been recently expanded to include variably protease-sensitive prionopathy, a rare sporadic condition recognised in a small number of patients, with certain features resembling Gerstmann–Sträussler–Scheinker syndrome (GSS) (Refs 5, 6, 7, 8). The genetic forms represent 10–15%

of all TSE cases and include familial CJD (fCJD), GSS and fatal familial insomnia (FFI) (Refs 9, 10). These diseases are inherited in an autosomal dominant fashion and are associated with more than 30 pathogenic mutations in the prion protein gene (*PRNP*) (Ref. 11). The infectious forms include variant CJD (vCJD), presumably resulting from dietary exposure to BSE (Ref. 12), iatrogenic CJD (iCJD) (Ref. 13), and kuru, an almost extinct disease described in cannibalistic tribes of New Guinea (Refs 14, 15, 16, 17). iCJD has been linked to therapeutic treatments with human pituitary hormones (growth hormone and gonadotropin), dura mater, cornea or pericardial grafts unknowingly sourced from CJD-afflicted individuals, and to rare neurosurgical procedures performed with inadequately decontaminated instruments previously used on CJD patients (Refs 13, 18). Recently, the transmission of vCJD has been reported in four instances through the therapeutic use of nonleukoreduced red blood cell concentrates (Refs 13, 19, 20, 21, 22).

Each form of human TSE results in a distinctive phenotype characterised by differences in the age of onset, variability in clinical symptoms, brain pathology and disease duration, and by different PrP<sup>TSE</sup> distribution and deposition patterns [reviewed in (Refs 3, 8, 10, 15, 16, 17, 23)]. The molecular mechanisms of TSE phenotypic heterogeneity are not fully understood, although polymorphisms in the host *PRNP* gene influence the phenotypic differences and individual susceptibility to certain forms of the disease (Refs 24, 25, 26, 27, 28, 29, 30, 31). These polymorphisms also partially explain the origin of biochemically distinct PrP<sup>TSE</sup> conformers substantiating the existence of prion strains in humans (Refs 32, 33, 34) and animals (Refs 35, 36, 37).

In animals, TSEs manifest, among other forms, as scrapie in sheep and goat, BSE (known to the general

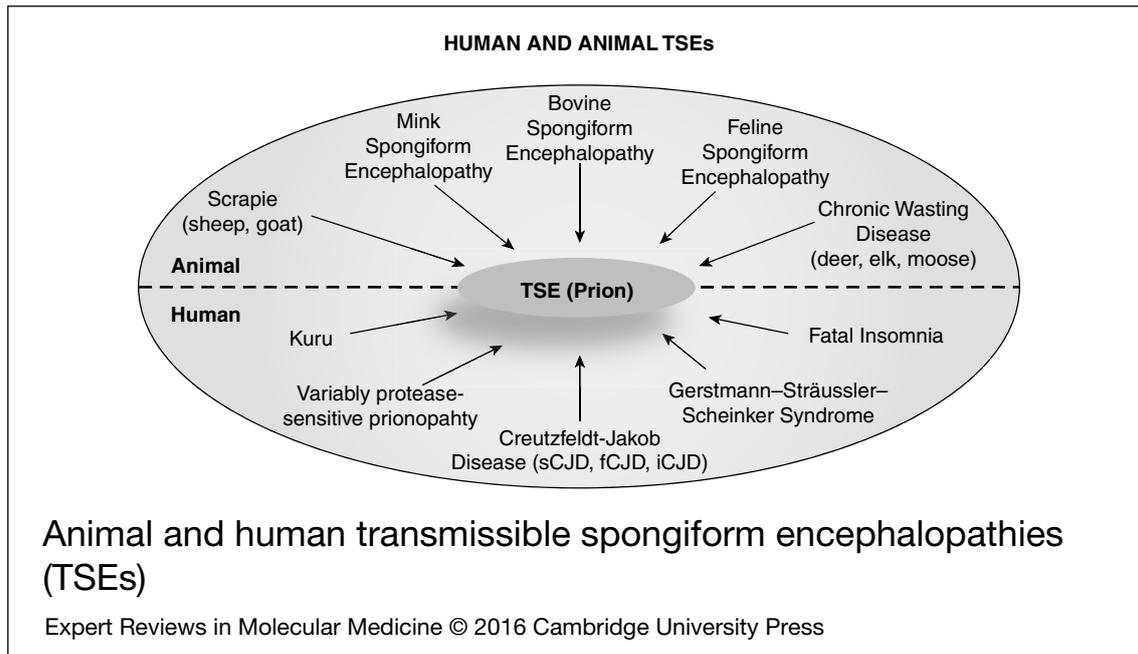


FIGURE 1.  
Animal and human TSEs.

public as ‘mad cow disease’) in cattle, and CWD in cervids (Fig. 1). Animal diseases are relatively easily transmitted within the same species, but cross-species transmissions have been documented as well. BSE is the only animal form of TSE that has been causatively linked to a disease in humans, vCJD (Refs 12, 38). For a complete review on animal prion disease, see Collinge (Ref. 39).

It is widely accepted that the causative agent of TSEs is the prion, which is mainly composed of a misfolded, insoluble, and proteinase K (PK)-resistant protein that is devoid of detectable informational nucleic acid, herein referred to as PrP<sup>TSE</sup> (also known as PrP<sup>Sc</sup>, PrP<sup>res</sup> or PrP<sup>d</sup>) (Refs 1, 2, 40). The exact mechanism(s) of PrP<sup>TSE</sup> generation are not fully understood. According to the current state of knowledge, the main event is the conformational change of PrP<sup>C</sup> into PrP<sup>TSE</sup>. This transition occurs under unknown circumstances and gives rise to multiple conformers exhibiting a range of strain-specific phenotypes in afflicted hosts (Ref. 41). Temporal and spatial deposition of PrP<sup>TSE</sup> coincides with a series of pathological events in the brain, resulting in spongiform degeneration, neuronal loss, and gliosis, which constitute the hallmarks of TSEs (Ref. 42). Prions utilise several routes of infection, which determine the length of the silent incubation period in an infected host. Usually, peripheral extra-neural exposures result in incubation phases that are longer than direct intracerebral routes. Factors and mechanisms underlying prion intra- and inter-species transmission leading to neurodegeneration are still under study, but significant progress has been made in the three decades following their discovery.

### PrP<sup>C</sup> function

PrP<sup>C</sup> is a sialoglycoprotein of 253 amino acids (human PrP<sup>C</sup>) encoded by a single gene. Post-translational processing in the endoplasmic reticulum (ER), results in the removal of an amino (N)-terminal signal sequence peptide (residues 1 to 22), and a carboxi (C)-terminal sequence for the attachment of a glycosyl phosphatidyl inositol (GPI) anchor to Ser-231 (Ref. 43). The N-terminal domain of the protein contains a repetitive sequence (residues 52–91) of eight amino acids, the so-called octapeptide repeats (PHGGGWGQ) that appear five times in most mammalian species, a neurotoxic domain or central region (CR) (residues 106–126), and a hydrophobic domain (residues 112–135). Additionally, PrP harbours a disulphide bridge linking residues Cys-179 and Cys-214, and two glycosylation sites at residues Asn-181 and Asn-197 (human PrP<sup>C</sup> numbering) (Refs 44, 45). Nuclear magnetic resonance spectroscopy analyses of full-length recombinant murine and hamster PrP<sup>C</sup> indicate that the secondary structure of the protein consists of a globular domain (residues 126–226) containing three  $\alpha$ -helices, two  $\beta$ -strands, and a short helix-like segment comprising residues 222–226, a flexible random-coiled like N-terminal tail spanning residues 23–125, and a disordered C-terminal region (residues 227–231) (Refs 44, 46, 47).

The physiological role of PrP<sup>C</sup> is still under debate, and defining its cellular role is complicated by the lack of major anatomical or developmental defects observed in early studies with PrP<sup>C</sup>-null (PrP<sup>-/-</sup>) mice generated after germline genetic ablation of PrP<sup>C</sup> expression (Refs 48, 49). Likewise, PrP<sup>-/-</sup> cattle produced by sequential gene-targeting showed

no physiological, immunological and reproductive abnormalities (Ref. 50). Additionally, genetically engineered *Prnp*<sup>+/-</sup> and *Prnp*<sup>-/-</sup> goats (Refs 51, 52), and even those that are devoid of PrP<sup>C</sup> as a result of a naturally occurring nonsense mutation (Ref. 53), presented normal development and behaviour. Moreover, transgenic mice generated by cell-specific targeted cre-mediated post-natal ablation of PrP<sup>C</sup> in neurons, showed no evidence of neurodegeneration or other histopathological changes for up to 15 months post-ablation (Ref. 54). However, certain alterations in physiological functions were reported in some *Prnp*<sup>-/-</sup> models. These included sleep disturbances, distorted circadian rhythm (Ref. 55), and abnormalities in synaptic transmission (specifically in cognition, olfactory physiology, and behaviour) [reviewed in (Refs 56, 57)]. Furthermore, one laboratory reported age-related defects in motor coordination and balance in PrP<sup>-/-</sup> mice; importantly, impaired mice displayed spongiform changes and reactive astrocytic gliosis in the brain, which usually accompany TSE pathology (Ref. 58). These findings suggested a plausible role for PrP<sup>C</sup> in neuroprotection during ageing. Electrophysiological studies pointed to a role for PrP<sup>C</sup> in modulating neuronal excitability. In these studies, PrP<sup>-/-</sup> mice exhibited long-term potentiation (LTP) impairment and reduced after-hyperpolarisation currents (Refs 59, 60). These findings were later confirmed in experiments involving post-natal removal of neuronal PrP<sup>C</sup> expression (Ref. 54).

The existing controversy in findings describing alterations in PrP<sup>-/-</sup> mice remains highly discussed, since variations in the genetic background of PrP<sup>-/-</sup> mouse models and PrP-flanking genes, rather than PrP<sup>C</sup> absence, may account for some of the observed phenotypes (Refs 61, 62). Overall, what appears to be an irrefutable phenotype in PrP<sup>-/-</sup> mice is resistance to infection with prions (Refs 63, 64, 65).

Many of the putative functions of PrP<sup>C</sup> are related to its cellular localisation. PrP<sup>C</sup> is attached to the outer leaflet of the plasma membrane through the GPI anchor (Ref. 43). In mammals, PrP<sup>C</sup> is expressed in various cell types throughout the body, with the highest levels reported in neurons (Refs 66, 67, 68, 69, 70, 71, 72). While the exact PrP<sup>C</sup> localisation in the cell is still debated, three major sites have been identified: plasma membrane, Golgi apparatus and early and late endosomes (Ref. 73). PrP<sup>C</sup> is mainly localised in cholesterol-rich microdomains, or lipid-rafts, at the plasma membrane (Refs 73, 74, 75). Since lipid rafts are platforms for signal transduction processes, it has been suggested that PrP<sup>C</sup> may trigger signalling pathways inside the cell (Ref. 76), with the resulting modulation of neuronal survival (Refs 77, 78) or neuritic outgrowth (Refs 78, 79). Several groups localised PrP<sup>C</sup> at the membrane of synaptic specialisations, including pre- and post-synaptic membranes, and on synaptic vesicles (Refs 80, 81, 82, 83, 84), further supporting its proposed role in neuronal synaptic transmission regulation

(Refs 59, 60). Additionally, PrP<sup>C</sup> was shown to interact with several proteins involved in synaptic release (Refs 85, 86) and with various ion channels (Ref. 87). In line with these observations, PrP expression in *Drosophila* resulted in synaptic vesicle optimisation and higher vesicle release efficiency, supporting a functional role for PrP<sup>C</sup> in protein signalling and synaptic plasticity (Ref. 88).

In addition to its subcellular localisation, PrP<sup>C</sup> has been detected in the cytosol in certain subpopulations of neurons in the hippocampus, neocortex and thalamus, but not in the cerebellum (Ref. 80). These neurons may play a significant role in the pathogenesis of prion diseases (Refs 80, 89). Differences in cytosolic PrP<sup>C</sup> distribution have never been addressed, but it has been suggested that cytosolic PrP<sup>C</sup> may have altered propensity for aggregation. Additional studies revealed that mammalian cells contain all co-factors required for cytosolic prion propagation and dissemination (Ref. 90). Intriguing is the fact that, unlike mammals, the expression of PrP<sup>C</sup> in the CNS of adult chickens was observed in dendrites and axons of neurons associated with certain sensory systems, but not in neuronal bodies or glial cells (Ref. 91).

Recent studies highlight zebrafish as a useful model for studying proteins implicated in neurodegenerative diseases, including PrP<sup>C</sup> (Ref. 92). Zebrafish express high levels of duplicated PrP homologue proteins, namely PrP-1 and PrP-2, in the developing and adult brain (Refs 93, 94). Both proteins are functionally related and share similarities to mammalian PrP<sup>C</sup> in terms of domain composition, the presence of two N-glycosylation sites, and their binding to the plasma membrane via a GPI anchor (Refs 92, 94, 95, 96). However, unlike findings in mammals, genetic silencing of PrP-1 or PrP-2 caused profound morphological defects in zebrafish embryonal development, with the knocking down of each protein affecting different stages of embryogenesis. Further studies revealed a role for PrP-1 in cell-to-cell adhesion, not only through homophilic interactions, but also by modulating the E-cadherin signalling cascade and regulating cell-to-cell communication in vivo (Refs 92, 96). Moreover, interaction between *Drosophila* Schneider 2 cells separately expressing mouse and fish PrP resulted in cell aggregation and activation of an intracellular signalling cascade leading to the modulation of E-cadherin, suggesting that PrP trans-interactions are highly conserved and can take place across a wide range of species (Ref. 92).

Numerous putative neuroprotective functions attributed to PrP<sup>C</sup>, including cell surface signalling, antioxidant and anti-apoptotic effects have been proposed [reviewed in (Ref. 97)]. Compelling evidence has been obtained for its role in myelination, autophagy regulation and trafficking of metal ions (Refs 98, 99, 100, 101, 102). Protein interaction analysis identified a subset of the ZIP (Zrt- Irt-like Protein) family of Zinc transporters as PrP<sup>C</sup> interaction partners.

Additional sequence analysis across a wide range of species within the chordate lineage, suggested that the prion gene family is phylogenetically derived from a ZIP-like ancestral molecule, providing an explanation to the functional role of PrP<sup>C</sup> in the trans-membrane transport of divalent cations (Ref. 103). PrP<sup>C</sup> is a copper (Cu<sup>2+</sup>)-binding protein that may play a role in Cu<sup>2+</sup> homeostasis by mediating Cu<sup>2+</sup> transport or sequestration (Refs 104, 105). It has been debated whether binding of Cu<sup>2+</sup> during PrP folding provides superoxide dismutase activity (SOD) on the protein (Refs 104, 106, 107) or if PrP<sup>C</sup> acts as an anti-oxidant by binding potentially harmful Cu<sup>2+</sup> ions, quenching free radicals generated as a result of Cu<sup>2+</sup> redox cycling (Ref. 108). A recent study performed with PrP<sup>C</sup>-deficient neuronal cells, suggested PrP<sup>C</sup> participates in anti-apoptotic and anti-oxidative processes by interacting with the stress inducible protein 1 (STI-1) to regulate SOD activation, reconciling previously discordant findings (Ref. 109).

The PrP<sup>C</sup> octapeptide repeat region has limited structural similarity to the B-cell lymphoma 2 (Bcl-2) homology domain 2 (BH2) of the family of apoptosis regulating Bcl-2 proteins (Ref. 97). Binding studies performed with the yeast two-hybrid system demonstrated a direct interaction between PrP<sup>C</sup> and the C-terminus of anti-apoptotic Bcl-2, but not of pro-apoptotic Bcl-2-associated X protein (Bax) (Refs 110, 111). In light of these findings, it was proposed that PrP<sup>C</sup> might assume the neuroprotective function of Bcl-2 proteins (Ref. 97). Indeed, hippocampal neurons isolated from PrP<sup>-/-</sup> mice were more susceptible to serum deprivation-induced apoptosis than their wild-type counterparts, and overexpression of either PrP<sup>C</sup> or Bcl-2, rescued this phenotype (Ref. 112). Experiments with human primary neurons (Ref. 113) and yeast models expressing mammalian prions (Ref. 114) provided further support to the neuroprotective effect of PrP<sup>C</sup> in pro-apoptotic Bax-mediated cell death. Expression in primary neurons of mutant PrP<sup>C</sup> harbouring a four octapeptide repeat deletion or the pathogenic substitutions T183A or D178N implicated in familial forms of TSE (Ref. 113) fully or partially abolished PrP<sup>C</sup> neuroprotective function. Furthermore, Bax-mediated neuronal loss was reported in Tg(PG14) mice expressing, in analogy to a human mutation, PrP<sup>C</sup> with a nine extra octapeptide repeat insertion that perhaps lost its physiological function because of misfolding and/or protein aggregation (Ref. 115). The finding that GPI attachment is not required for PrP<sup>C</sup> cyto-protective function remains unexplained (Refs 113, 114). The role of PrP<sup>C</sup> in apoptosis will be further discussed.

The finding that PrP<sup>-/-</sup> mice show subtle abnormalities in immune system function, and that PrP<sup>C</sup> expression on bone marrow long-term hematopoietic stem cells seems to be important for self-renewal (Ref. 116), highlighted a physiological function for PrP<sup>C</sup> outside the CNS. PrP<sup>C</sup> was also implicated in

T-cell cytokine response, where *Prnp* mRNA up-regulation and increased PrP<sup>C</sup> expression followed T-cell activation (Refs 117, 118), while PrP<sup>C</sup> expression ablation led to reduced induction of helper T-cell cytokine production (Refs 118, 119). Additional studies, suggested a role for PrP<sup>C</sup> at the immunological synapse by showing that absence of PrP<sup>C</sup> on antigen presenting dendritic cells resulted in a significant reduction of proliferative potential of responding T-cells (Ref. 120).

#### *PrP<sup>C</sup> interaction with proteins implicated in AD*

The membrane localisation of PrP<sup>C</sup> places this protein in close proximity with the amyloid precursor protein (APP) and other proteins involved in amyloid- $\beta$  (A $\beta$ ) processing. A putative role for PrP<sup>C</sup> in AD has been recently reviewed (Ref. 121).

In contrast to the earlier work presented by Parkin (Ref. 122) indicating a protective role for PrP<sup>C</sup>, Lauren et al suggested that PrP<sup>C</sup> mediates oligomeric A $\beta$  (oA $\beta$ ) neurotoxicity. One manifestation of this role is that PrP<sup>C</sup> is required for oA $\beta$ -induced LTP impairment (Ref. 123), a finding later confirmed by others (Ref. 124). Further studies provided evidence for the pathophysiological role of PrP<sup>C</sup> in mediating several other toxic effects of oA $\beta$ , including synaptic plasticity disruption (Refs 124, 125), axonal degeneration of serotonergic neurons, synapse loss and deficits in spatial learning and memory (Ref. 126), neuronal cell death (Ref. 127) and synapse damage (Ref. 128). Alternative studies assert that PrP<sup>C</sup> is not required for A $\beta$ -dependent synaptic depression, LTP impairment (Refs 129, 130, 131) or premature mortality, and abnormal neural network activity (Ref. 132). Others showed that, overexpression of PrP<sup>C</sup> prevents A $\beta$ <sub>1-40</sub>-induced spatial learning and memory deficits by modulating programmed cell death pathways (Ref. 133).

PrP<sup>C</sup> may play a role in neurotoxic signalling pathways by sensitising cells to toxic effects of  $\beta$ -sheet-rich conformers of different origins (Ref. 134). Other mechanisms have been described involving additional interacting molecules. One study showed that A $\beta$  neurotoxicity depends on interactions between Cu<sup>2+</sup>, PrP<sup>C</sup> and N-methyl-D-aspartate receptor (NMDA) (Ref. 135). Others revealed that neuronal impairment occurs via Fyn activation through oA $\beta$  binding to synaptic PrP<sup>C</sup> (Ref. 136).

While discrepancies in the contribution of PrP<sup>C</sup> to AD pathogenesis may have arisen from differences in the animal models and A $\beta$  preparations employed, all these studies suggest that the prion protein binds oA $\beta$ . Further investigations will clarify the interrelationship of both proteins and neurodegeneration.

#### **Neurodegeneration pathways in prion disease**

Owing to the temporal and spatial coincidence of PrP<sup>TSE</sup> accumulation and the appearance of the first neurodegenerative changes in the brain, it has been suggested that either the loss of a critical biological

function of PrP<sup>C</sup> or the acquisition of toxic properties upon conversion into PrP<sup>TSE</sup> triggers neurodegeneration and disease.

#### *Loss of PrP<sup>C</sup> function*

PrP<sup>C</sup> has been implicated in several mechanisms leading to neuronal protection from oxidative stress or other types of pro-apoptotic insults (Refs 97, 137). Therefore, it seems logical that conversion of PrP<sup>C</sup> into PrP<sup>TSE</sup> may render the former unable to perform its normal biological function, leading to neurodegeneration. This theory is strongly challenged, however, by compelling data showing normal embryonic development and absence of major anatomical or functional phenotypes in mammals where PrP<sup>C</sup> expression has been permanently or conditionally knocked out (Refs 48, 49, 50, 51, 52, 53, 54).

#### *Gain of toxic function*

PrP<sup>TSE</sup> is considered the surrogate marker of prion diseases. It accumulates in various regions of the brain, either in a diffused pattern or in the form of aggregates of different types depending on the strain of the agent and the host species (Refs 34, 138). The co-occurrence of PrP<sup>TSE</sup> accumulation and spongiform changes in the brains of the majority of TSE patients, and in the brains of naturally and experimentally infected animals, prompted investigators to attribute a toxic function to PrP<sup>TSE</sup>. This assumption was supported by *in vitro* studies reporting neurotoxicity of micromolar concentrations of short PrP<sup>C</sup> peptides, encompassing residues 106–126 of human PrP<sup>C</sup> (Ref. 139), and nanomolar concentrations of purified PrP<sup>TSE</sup> (Ref. 140).

While these data suggest a direct involvement of PrP<sup>TSE</sup> as the toxic agent in TSE pathogenesis, several lines of evidence do not support a causative relationship. Some forms of TSE, including FFI, have very restricted brain pathology with little or no spongiform change or detectable PrP<sup>TSE</sup> (Ref. 141). PK-resistant PrP<sup>TSE</sup> has been identified in non-CJD brains suggesting that the accumulation of PrP<sup>TSE</sup> is probably not neurotoxic (Refs 142, 143). Dissociation between PrP<sup>TSE</sup> and neurodegeneration has also been reported in several cases of natural and experimental human prion disease, where neuropathological changes occurred in the absence or with very limited PrP<sup>TSE</sup> accumulation (Refs 144, 145, 146, 147). A number of studies have reported no correlation between PrP<sup>TSE</sup> deposition and neurodegeneration in the brains of infected mice expressing half the normal levels of PrP<sup>C</sup> (Refs 148, 149, 150, 151). Bueler and colleagues showed that although these mice accumulated PrP<sup>TSE</sup> and infectivity titres similar to those found in clinically sick, wild-type animals, they had a significant delay in disease onset and progression indicative of relative resistance to the toxic effects of prions (Ref. 150). Likewise, it is well documented the existence of sub-clinical carriers of prion infection, with wild-type mice living a typical lifespan despite harboring

PrP<sup>TSE</sup> titres similar to mice at the end-stage of the disease (Refs 152, 153, 154, 155, 156, 157). Compelling experimental evidence against the direct neurotoxic effect of PrP<sup>TSE</sup> was first obtained in the seminal studies by Aguzzi and colleagues (Refs 158, 159) who showed that intra-cerebral injection of prions in PrP<sup>C</sup>-null mice with grafted neural tissue from a mouse overexpressing PrP<sup>C</sup>, resulted in severe spongiform changes, infectivity, and PK-resistant PrP<sup>TSE</sup> in the grafts. However, while in some instances PrP<sup>TSE</sup> was found in brain areas outside the graft, these regions were spared from neurodegenerative changes (Refs 158, 159). Work by Mallucci et al. (Ref. 160) provided additional proof against a direct role of PrP<sup>TSE</sup> in neurotoxicity. By specifically knocking down PrP<sup>C</sup> expression in mouse neurons after established prion neuroinvasion, they showed reversion of early spongiform changes and prevention of disease progression, despite continuous accumulation of PrP<sup>TSE</sup> in the neuropil (the latter coming from the conversion of PrP<sup>C</sup> produced in nonneuronal cells) (Ref. 160).

Multiple studies suggested that soluble oligomeric species, intermediates in the formation of protease-resistant PrP<sup>TSE</sup>, are the pathogenic entity rather than insoluble PrP<sup>TSE</sup> aggregates (Refs 161, 162, 163, 164, 165, 166). The idea has been further developed that PrP<sup>TSE</sup> replication and neurotoxicity occur in two separate phases, where the production of a neurotoxic species (PrP<sup>L</sup>) is catalysed by PrP<sup>TSE</sup>. According to this model, when PrP<sup>TSE</sup> amplification saturates, the autocatalytic production of infectivity (phase 1) switches into a toxic pathway (phase 2) leading to the formation of toxic PrP species. Importantly, the formation of PrP<sup>L</sup> is linearly dependent on PrP<sup>C</sup> concentration (Refs 34, 148, 151).

#### *Subversion of PrP<sup>C</sup> function*

The experimental observation that PrP<sup>C</sup> expression at the neuronal membrane is required to transduce a toxic signal inside the cell (Refs 54, 167) established the molecular basis of a new mechanism to explain neurotoxicity in prion diseases: subversion of PrP<sup>C</sup> function (Ref. 168), whereby interaction between PrP<sup>C</sup> and PrP<sup>TSE</sup>, or intermediate species, subverts or modifies the normal function of PrP<sup>C</sup>, triggering a toxic signal inside the cell (Fig. 2). While the prevailing data argue for the necessity of neuronal PrP<sup>C</sup> expression for neurotoxicity, some challenging evidence still exists (Refs 54, 149, 165, 166, 167, 169, 170). Studies by Zhou et al. identified a monomeric, highly  $\alpha$ -helical form of PrP<sup>C</sup>, the so-called toxic PrP (TPrP), as the most neurotoxic PrP species *in vitro* and *in vivo*. TPrP was generated *in vitro* by size fractionation following dilution refolding of full-length mouse recombinant PrP<sup>C</sup>, and it was shown to elicit autophagy, apoptosis and a molecular signature similar to that observed in the brains of prion-infected animals (Ref. 170). TPrP was toxic to PrP<sup>-/-</sup> mouse-

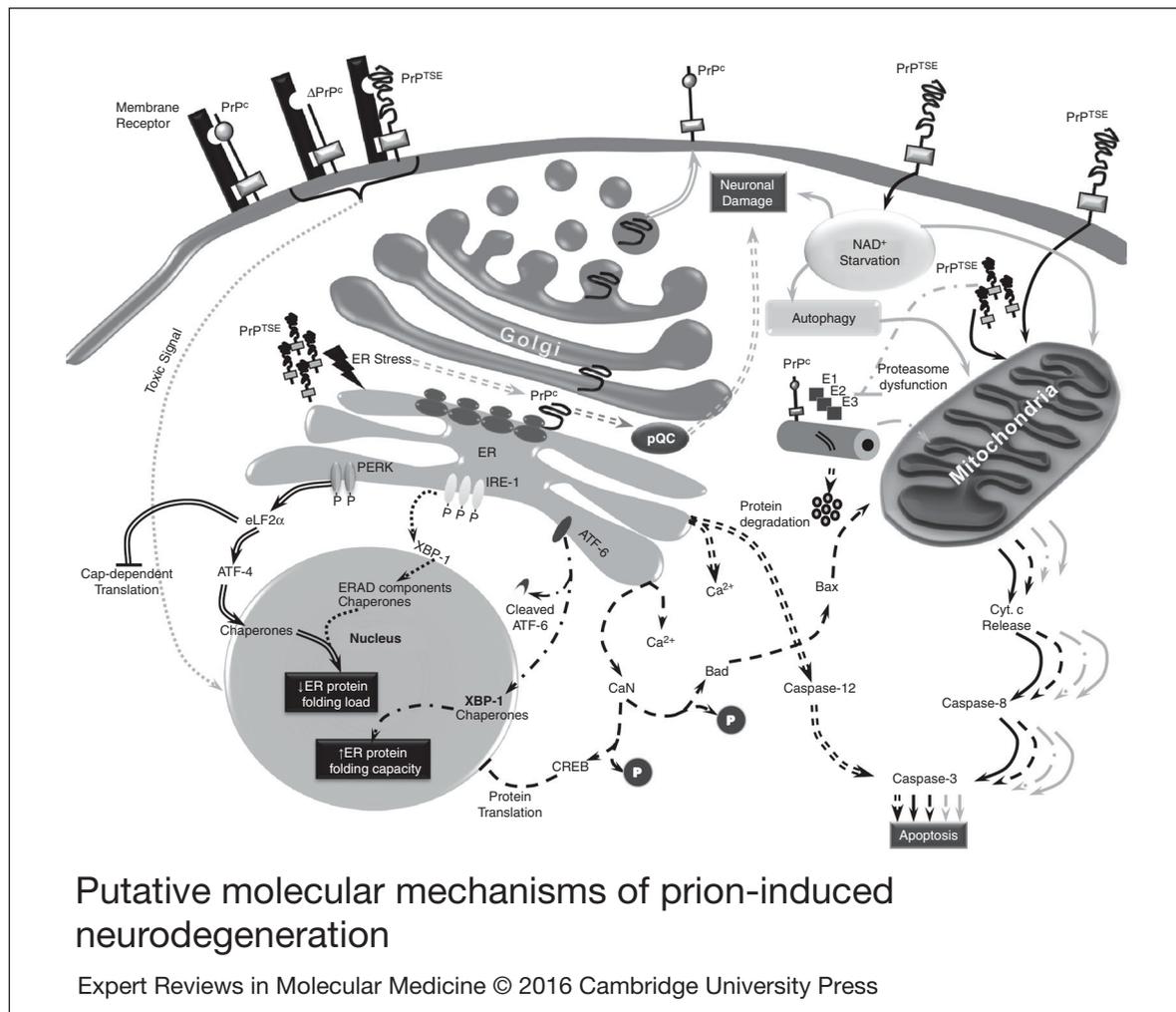


FIGURE 2.

Putative molecular mechanisms of prion-induced neurodegeneration. Schematic representation of different mechanisms by which PrP<sup>C</sup> misfolding and PrP<sup>TSE</sup> accumulation may result in cellular death and neuronal damage. PrP<sup>C</sup> synthesis: solid grey double lines. Subversion of function/Excitotoxic stress: dotted grey line. The mitochondrial pathway of apoptosis: solid black line. Endoplasmic reticulum stress-induced apoptosis: dotted black double lines. Endoplasmic reticulum stress: dashed black line. The preemptive quality control (pQC) pathway: dotted grey double lines. The unfolded protein response, IRE-1 arm: dotted black line. The unfolded protein response, PERK arm: solid black double lines. The unfolded protein response, ATF-6 arm: dash-dotted black line. The ubiquitin-proteasome system (UPS) and the aggresome: dash-dotted grey line. Normal PrP<sup>C</sup> degradation by the proteasome: dash-dotted black double lines. NAD<sup>+</sup> starvation: solid grey line. Cyt. C: Cytochrome C, E1: Ubiquitin activating enzyme, E2: Ubiquitin conjugating enzyme, E3: Ubiquitin protein ligase. —> Activates route. —| Inhibits route.

derived hippocampal neurons *in vitro*, supporting the hypothesis that endogenous neuronal PrP<sup>C</sup> is not required to propagate a toxic signal inside the cell (Ref. 170). However, even if TPrP toxicity does not rely on membrane-bound PrP<sup>C</sup> expression, experimental evidence suggests that it should be generated within neurons, given that post-natal ablation of PrP<sup>C</sup> expression in these cells reverses early neurodegenerative changes and prevents disease progression in mice, even though glial replication and accumulation of PrP<sup>TSE</sup> continues (Ref. 54). Indeed, growing evidence indicates that prion-induced pathology comprises cell-autonomous mechanisms, resulting in cellular dysfunction and neurodegeneration, and noncell-autonomous processes leading to prion spread (Ref. 171).

Studies performed with Tg44 transgenic mice expressing PrP<sup>C</sup> that lacks the GPI membrane

anchoring signal (GPI<sup>-</sup>-PrP<sup>C</sup>) provided additional supporting evidence to the dissociation between prion replication and neurotoxicity (Refs 149, 169, 172). In anchorless-PrP<sup>C</sup> transgenic mice, about 90% of GPI<sup>-</sup>-PrP<sup>C</sup> is secreted, the rest appearing in the ER and Golgi complex, but not on the plasma membrane. Scrapie infection of Tg44 mice resulted in a substantially different disease than that observed in wild-type mice, with an incubation period, clinical signs and neuropathological abnormalities characteristic of cerebral amyloid angiopathy (CAA) (Refs 149, 169, 173). Spongiform grey matter degeneration was minimal or not present in the brains of diseased mice, and this feature was preserved through subsequent multiple passages in Tg44. However, the brains of these mice contained large deposits of amyloid PrP<sup>TSE</sup> and high levels of infectivity that caused classical grey matter

spongiosis with PrP<sup>TSE</sup> accumulation in wild-type mice (Ref. 169). Experimental studies involving peripheral scrapie infection of Tg44 mice revealed a crucial role for membrane-bound PrP<sup>C</sup> in neuroinvasion and neuronal PrP<sup>TSE</sup> spread (Ref. 173), and for the induction of a typical TSE pathogenic process (Ref. 149). Interestingly, whereas the neuropathogenic processes found in Tg44 mice i.c. injected with prions were distinct from typical prion disease, they were reminiscent of changes found in familial forms of TSEs associated with STOP mutations at codons 145, 163 and 226 of *PRNP* (Refs 174, 175, 176). These changes involved dense amyloid PrP<sup>TSE</sup> plaque deposits with CAA, but without grey matter spongiosis.

Because prion diseases are a group of diverse disorders characterised by different disease phenotypes and different pathological features (Ref. 177), it is likely that neuropathogenesis in these disorders is triggered by different mechanisms, the majority of which depend on PrP<sup>C</sup> anchoring to the neuronal membrane. In typical prion disease, neuronal PrP<sup>C</sup> is required for neuroinvasion and for PrP<sup>TSE</sup>-mediated neurotoxic membrane interactions (Ref. 149). Neurotoxicity independent of neuronal PrP<sup>C</sup> expression was observed in Tg44 mice after i.c. injection of scrapie, where neuropathogenesis was likely the result of tissue distortion by amyloid plaques, obstruction of interstitial fluid flow, and vascular occlusion triggered by the accumulation of PrP<sup>TSE</sup> amyloid within basement membranes and interstitial space between neurite and glial processes. Proteomic analysis of CAA and nonamyloid TSE disease phenotypes in mice revealed similarities and differences in the mechanism of pathogenesis (Ref. 178). Following scrapie infection with the scrapie strain RML, the brains of wild-type and Tg44 mice showed evidence of a neuroinflammatory response and complement activation. However, ER-associated degradation (ERAD) and mitochondrial induced apoptosis pathways were implicated only in wild-type animals exhibiting nonamyloid disease phenotype, whereas metal binding and synaptic vesicle transport were more profoundly disrupted in Tg44 mice with PrP<sup>TSE</sup>-CAA accumulation (Ref. 178). A similar unique mechanism may be responsible for the phenotypic differences observed in certain forms of human TSE (Ref. 149).

### **Molecular and cellular mechanisms of neuronal death**

With just few exceptions, TSEs are characterised at the neuropathological level by various degrees of spongiform vacuolation of the neuropil, accompanied by neuronal cell loss and gliosis, which together constitute the classic neuropathological triad of TSEs (Ref. 42). Ultrastructural studies revealed that typical 'spongiform vacuoles' develop within neuronal elements or within myelinated axons or myelin sheaths. The origin of these vacuoles is still debated, but the prevailing view attributes their occurrence to autophagy rather

than to abnormalities in membrane permeability leading to increased water retention (Refs 137, 179).

### *Autophagy in prion diseases*

Basal autophagy plays an important role in maintaining cell homeostasis and physiological function. It is characterised by the formation of cytoplasmic autophagic vacuoles that fuse with lysosomes to degrade and recycle the vesicular content. This is a tissue-specific, tightly regulated process mediated via the lysosomal degradation pathway. Alternatively, autophagy induced by various cellular insults is recognised as one of the three mechanisms of programmed cell death in eukaryotes (Ref. 180).

Autophagic vacuoles have been identified in several neurodegenerative diseases, including various forms of TSEs (Refs 181, 182, 183). Since, under normal conditions, autophagy takes place at low levels in the CNS an increase in the number of autophagosomes in prion-infected brains was interpreted by some as cause of neurodegeneration (Refs 170, 184, 185). However, based on recent studies providing evidence that PrP<sup>C</sup> may play a role in autophagy regulation in neurons, and since the absence of PrP<sup>C</sup> expression resulted in autophagy up-regulation (Ref. 102), it can be argued that loss or subversion of PrP<sup>C</sup> function during prion infection may trigger a similar response.

Although some authors consider autophagosomes to be part of the mechanism leading to neuronal death (Refs 170, 184), a debate continues as to whether they play a neuroprotective role through degradation of intraneuronal deposits of PrP<sup>TSE</sup>. Early studies showed impaired PrP<sup>TSE</sup> aggregation and protection against oxidative damage following trehalose treatment of prion-infected cells (Ref. 186). Schatzl and colleagues advanced these observations and provided direct *in vitro* evidence of autophagy-induced PrP<sup>TSE</sup> degradation (Ref. 181). Pharmacological treatment of prion-infected neuronal and non-neuronal cells, with the autophagy-inducing agents imatinib, rapamycin, lithium and trehalose, increased cellular clearance of PrP<sup>TSE</sup> (Refs 187, 188, 189). Early administration of imatinib after peripheral inoculation of prions in mice delayed disease onset and accumulation of PrP<sup>TSE</sup> in the CNS (Ref. 190). However, neither intraperitoneal nor intracerebroventricular delivery of the drug showed beneficial effect on PrP<sup>TSE</sup> clearance in the CNS (Ref. 190). Likewise, the survival of mice i.p. infected with scrapie was not influenced by trehalose treatment despite the delayed appearance of PrP<sup>TSE</sup> in the spleen (Ref. 187).

Recent observations derived from the pharmacological manipulation of autophagy with either autophagy-enhancing or -inhibiting drugs showing no changes on the time course or amplitude of neuronal death in response to TPrP exposure, strongly suggested that the observed autophagy in protein misfolding diseases is a secondary mechanism in the neurodegenerative process (Ref. 191). Consistent with this idea are in

vivo studies where Tg(PrP-A116V) mice, a model of GSS, were chronically treated with i.p. injections of rapamycin. Drug treatment led to a dose-dependent delay in disease onset, a reduction in symptom severity, and improved survival concomitant with increased levels of the autophagy-specific marker LC3-II (microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate), reduced levels of insoluble PrP-A116V, and a near to complete absence of PrP amyloid plaques in the brain. However, despite the reported reduction in amyloid burden, these mice eventually reached terminal levels of motor impairment and succumbed to the disease (Ref. 192).

#### *Role of apoptosis in TSEs*

Not all histopathological changes identified in TSE-affected brains can be attributed to the activation of cellular autophagy. Indeed, several studies conducted in natural disease and experimental models of TSEs observed DNA fragmentation and the activation of several caspases, indicating that neuronal death may occur via apoptotic pathways (Refs 193, 194, 195).

*The mitochondrial pathway.* This was identified in experimental models where aggregated PrP peptides, like PrP106-126, or recombinant mutant PrP were used (Refs 196, 197, 198). These toxic forms of PrP caused alterations in the mitochondrial membrane leading to mitochondrial stress, cytochrome c release, caspase activation, and ultimately, neuronal death (Fig. 2, solid black line). Early findings suggested that cerebellar granule neurons (CGNs) in Tg(PG14) mice died via a Bax-dependent process (Refs 115, 199). However, crossing these animals with Bax<sup>-/-</sup> mice had no effect on disease onset and duration. Additionally, despite Bax inactivation significantly inhibited apoptotic death of CGNs, it did not rescue synaptic degeneration and did not prevent neurological disease. Instead, these findings supported synaptic degeneration, and not apoptotic neuronal loss, as the primary pathologic event contributing to the clinical signs observed in this model (Ref. 115). Experiments with Bax deficient mice provided additional evidence against the primary role of proapoptotic Bax in neuronal cell death. Inoculation of Bax<sup>-/-</sup> and wild-type control mice with mouse-adapted BSE prions showed no differences in terms of PrP<sup>TSE</sup> accumulation, neurodegeneration, disease onset and clinical signs, providing compelling evidence that Bax-mediated cell death was not involved in the pathological mechanism induced by BSE (Ref. 200). Nevertheless, cleaved caspase-3 and -9 were found in the brains of Bax<sup>-/-</sup> mice, suggesting that apoptosis may occur through an alternative mechanism in TSEs of infectious origin. These observations were consistent with previous findings of apoptotic features in the brains of wild-type mice infected with RML, in the absence of Bax upregulation (Ref. 201). More recently, proteomic analyses of

wild-type mice injected with the same agent revealed upregulation of proteins involved in cell death and survival; in particular, in the levels of proteins associated with the mitochondrial inner membrane, proteins associated with the ubiquitin/proteasome pathway, and proteins involved in the ERAD (Ref. 178), but not in the brains of RML-infected Tg44 mice. This evidence further supported the notion that distinct pathways may be activated in TSEs of different aetiology.

*The endoplasmic reticulum pathway.* ER stress has been recently discovered as a novel apoptotic-regulatory pathway, with implications in  $\beta$ -amyloid cytotoxicity (Ref. 202). Stress of the ER results from changes in Ca<sup>2+</sup> homeostasis or accumulation of aggregated proteins. Either situation will induce Ca<sup>2+</sup> release from the ER and activation of the ER-membrane-resident caspase-12. Following activation, caspase-12 is released into the cytoplasm where it activates downstream caspases of the apoptotic response (Fig. 2, dotted black double lines). Additionally, Ca<sup>2+</sup> regulates calcineurin (CaN), a type 2 phosphatase involved in synaptic function, memory and cell death (Ref. 203).

Dysregulation of intracellular Ca<sup>2+</sup> balance has been described in a number of neurodegenerative proteinopathies, including TSEs (Ref. 140). Early studies identified ER stress and caspase-12 activation in murine neuroblastoma (N2a) cells infected with highly purified prion preparations. Similar changes were observed in prion-infected mice as well as in brains of patients affected with vCJD and sCJD (Ref. 140). But, even if these findings initially suggested the involvement of a caspase-12-dependent apoptotic pathway in naturally occurring prion diseases, infectivity studies with caspase-12 knockout and wild-type mice revealed identical disease onset and progression, irrespective of caspase-12 expression, arguing against its role in neurotoxicity (Ref. 204).

Further investigations on the role of ER stress and Ca<sup>2+</sup> homeostasis in neurodegeneration suggested that PrP<sup>TSE</sup> accumulation caused synaptic dysfunction and neuronal death via CaN activity dysregulation (Ref. 205). This hypothesis stemmed from in vitro studies with N2a cells that were either treated with brain-derived PrP<sup>TSE</sup>, or engineered to overexpress mutant PrP forms associated with familial TSEs. Thus, pharmacological manipulation of Ca<sup>2+</sup> homeostasis in scrapie-infected N2a cells led to ER stress (Ref. 206). Moreover, treatment of cells with brain-derived PrP<sup>TSE</sup> induced upregulation of the unfolded protein response (UPR)-responsive chaperones glucose regulated protein (Grp) 58, Grp78, and Grp94, which was indicative of ER stress. Additionally, overexpression of SERCA (ER-Ca<sup>2+</sup> ATPase) in cells made them highly susceptible to PrP<sup>TSE</sup>-induced cell death. The study of N2a cells overexpressing murine PrP carrying mutations associated with FFI (PrP<sup>D177N/Met128</sup>) (murine PrP numbering) or GSS (PrP<sup>PG14</sup>), or neurotoxic transmembrane

forms, showed decreased ER  $\text{Ca}^{2+}$  content upon treatment with  $\text{Ca}^{2+}$  agonists (Ref. 206). According to this model, hyperactivation of CaN, because of increased cytosolic levels of  $\text{Ca}^{2+}$ , results in the dysregulation of the pro-apoptotic molecule Bcl-2-associated death promoter (Bad), and the transcription factor cAMP response element-binding (CREB), among other targets. Upon dephosphorylation, Bad interacts with Bax causing mitochondrial stress and apoptosis. Dephosphorylated CREB cannot translocate into the nucleus, where it regulates the transcription of proteins involved in synaptic plasticity, resulting in synaptic degeneration (Ref. 207) (Fig. 2, dashed black line). Consistently, disruption in the expression of proteins involved in  $\text{Ca}^{2+}$  homeostasis and synaptic vesicle transport were also found in Tg44 mice with CAA (Ref. 178).

#### *Other mechanisms of neurodegeneration*

The studies described above suggest that autophagy and apoptosis are rather a secondary consequence of prion-induced neurodegeneration. To better understand the mechanism behind this process many laboratories have hypothesised alternative pathways triggering neurodegeneration based on other pathophysiological changes observed in the cell.

**Excitotoxic stress.** Important information about PrP<sup>C</sup> function and prion neurotoxicity has been gathered from the analysis of phenotypic variations produced by several PrP<sup>C</sup> deletion mutants in transgenic mice. It has been repeatedly shown that deletions within the N-terminal half of PrP<sup>C</sup> cause massive neuronal death in transgenic mice (Refs 208, 209, 210). This phenotype was rescued in a dose-dependent manner by co-expression of full-length PrP<sup>C</sup>. Therefore, it has been suggested that wild-type and truncated PrP<sup>C</sup> bind to a common molecular target eliciting two different responses in the cell: while truncated-PrP<sup>C</sup> binding triggers a toxic signal, binding of wild-type PrP<sup>C</sup> restores a nontoxic physiological function (Ref. 211) (Fig. 2, dotted grey line).

Transgenic mice expressing PrP<sup>C</sup> harbouring a deletion within the highly conserved CR of the protein, residues 105–125 (PrP $\Delta$ CR) (murine PrP numbering), have been generated in an attempt to define the PrP<sup>C</sup> sequence determining neurotoxicity (Ref. 209). When PrP $\Delta$ CR was expressed on the *Prnp*<sup>-/-</sup> background, mice died within the first week of life, whereas co-expression of wild-type PrP<sup>C</sup> alleviated the phenotype in a dose-dependent fashion. Neuropathological examination of mice co-expressing PrP $\Delta$ CR and one *Prnp* allele revealed dramatic degeneration of CGNs and vacuolation of white matter regions, features observed in TSEs (Ref. 212). Biochemical and morphological analyses could not attribute CGN death to apoptosis or autophagy, since no activation of caspases 3 or 8, or increased levels of the autophagy marker LC3-II were detected in these animals. Instead, degenerating

CGNs displayed a cytoplasmic morphology reminiscent of certain forms of excitotoxic neuronal death characterised by heterogeneous condensation of the nuclear matrix without formation of discrete chromatin masses (Ref. 212). The same morphology was present in the neurons of transgenic mice expressing a different deletion mutant, PrP $\Delta$ 32–134. These findings suggested common neurotoxic mechanisms for PrP<sup>C</sup> proteins missing the CR (Ref. 212) (Fig. 2).

**The preemptive quality control (pQC) system.** To further explain the necessity of a continuous supply of PrP<sup>C</sup> for neurotoxicity, Rane and colleagues, advancing an original idea by Orsi et al. (Ref. 213), proposed that the alteration of the cellular metabolism resulted in the synthesis of a neurotoxic form of PrP<sup>C</sup> (Ref. 214). Given the implication of this pathway in PrP<sup>TSE</sup> pathogenesis (Ref. 140), the authors highlighted chronic ER stress as an example of altered cellular metabolism, and suggested the existence and involvement of the pQC pathway (Refs 214, 215).

There are three mechanisms by which proteins may be degraded in the cytosol: failed targeting, retrotranslocation and pQC. However, only the pQC pathway is activated under ER stress. There is no evidence that PrP<sup>C</sup> is synthesised in the cytosol or is retrotranslocated from the ER during normal and stress conditions (Ref. 216). In light of these data, it has been suggested that PrP<sup>TSE</sup> accumulation triggers ER stress. Consequently, PrP<sup>C</sup> is no longer translocated into the ER but rapidly degraded by the pQC pathway. Persistent, accelerated routing of PrP<sup>C</sup> through the pQC results in neuronal damage and corresponding clinical symptoms by an unknown mechanism (Ref. 214) (Fig. 2, dotted grey double lines).

Since the model did not fully recapitulate all clinical and histopathological changes found in prion diseases, it was proposed that several pathways lead to neurodegeneration in these disorders, including increased production of transmembrane forms of PrP (C<sup>tm</sup>PrP), decreased proteasome activity, or generation of a toxic intermediate during prion conversion (Ref. 214).

**The UPR.** UPR activation is a cellular response to reestablish homeostasis by synthesis of properly folded proteins. It affects the expression of chaperones, enhances degradation of unfolded and mutated proteins, and inhibits protein synthesis (Ref. 217). When the concentration of unfolded proteins in the lumen reaches a threshold, a set of intracellular signal transduction pathways involving three transmembrane ER-resident signalling components, namely inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), is activated. Prolonged activity of the UPR is indicative of chronic ER stress and results in cellular apoptosis (Refs 217, 218).

IRE1, a bifunctional transmembrane kinase-endoribonuclease protein, initiates the nonconventional splicing of mRNA to propagate the UPR signal (Refs 217, 219). Activated IRE1 facilitates generation of an active form of the X-box binding protein 1 (XBP-1), a UPR-specific transcription factor that enhances the transcription of UPR genes involved in protein quality control, and activates ERAD among other targets (Ref. 220) (Fig. 2 dotted black line). While early *in vitro* studies in N2a cells indicated a protective role for UPR activation against PrP<sup>C</sup> misfolding under ER stress mediated by XBP-1 (Ref. 219), infection of conditional XBP-1 knockout mice with prions demonstrated that ablation of this gene had no effect on neuronal function and prion pathogenesis *in vivo*, providing compelling evidence against the hypothesised neuroprotective role of the XBP-1 branch of the UPR (Ref. 221).

Later studies in PrP<sup>C</sup> overexpressing Tg37 MloxP transgenic mice explored the involvement of the arm of the UPR responsible for translational control. This mechanism is directed by PERK phosphorylation of the ubiquitous eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (Fig. 2, solid black double lines) (Ref. 222). Upon ER stress, PERK aggregates and phosphorylates itself and eIF2 $\alpha$  (PERK-P and eIF2 $\alpha$ -P). Phosphorylation inactivates eIF2 $\alpha$ , and inhibits mRNA translation, therefore reducing the protein load in the ER to alleviate ER-stress (Ref. 217). However, sustained elevation of eIF2 $\alpha$ -P leads to a decline in global translation rates and loss of synaptic proteins, contributing to neuronal death. Importantly, increased levels of PERK-P and eIF2 $\alpha$ -P were detected throughout the course of the disease in RML-infected Tg37 mice (Ref. 222). Reduction of eIF2 $\alpha$ -P levels by lentiviral-induced overexpression of GADD34, an eIF2 $\alpha$ -P-specific phosphatase, or inhibition of PrP<sup>C</sup> expression by anti-PrP shRNA, rescued the pathologic phenotype and significantly increased mouse survival (Ref. 222). In contrast, pharmacological inhibition of eIF2 $\alpha$ -P dephosphorylation further enhanced neurotoxicity and significantly accelerated the disease (Ref. 222). These data strongly supported the conclusion that chronic UPR stress, with persistent expression of eIF2 $\alpha$ -P and continuing inhibition of protein synthesis, leads to synaptic failure, spongiosis and neuronal loss in TSEs (Ref. 222).

*The ubiquitin–proteasome system (UPS) and the aggresome.* Ubiquitination is an efficient system for targeting cellular proteins for degradation. However, impairment of the proteasomal machinery can result in atypical ubiquitination of proteins and accumulation of ubiquitinated proteasomal substrates. Aberrations in the regulation of the UPS have been associated with a wide range of neurodegenerative diseases (Refs 223, 224). The 26S proteasomal complex comprises a 20S proteolytic core complexed at one or both ends with a 19S regulatory complex. The 20S proteasome is

composed of 14  $\alpha$  and 14  $\beta$  subunits arranged in four stacked rings, the two outer rings consisting of 7  $\alpha$  units and the two inner rings of 7  $\beta$  subunits each. This organisation gives the 20S proteasome the appearance of a hollow barrel. Under physiological conditions, a small fraction of PrP<sup>C</sup> undergoes ubiquitination and proteasomal degradation (Ref. 225). Positive ubiquitin staining of proteins was found in brains of CJD patients and experimental scrapie-infected mice (Refs 226, 227). In humans afflicted with CJD, extensive aggregated deposits of PrP<sup>TSE</sup> frequently co-localise with ubiquitin, while fine granular deposits do not appear to be ubiquitinated (Ref. 228). The notion that in scrapie-infected mice protein ubiquitination increased during infection while proteasomal activity declined, led to the suggestion that ubiquitination occurs after the formation of protease-resistant PrP<sup>TSE</sup> (Ref. 229) (Fig. 2, dash-dotted grey line). Studies using scrapie-infected N2a cells showed that PrP<sup>TSE</sup> co-localised with the cytosolic marker heat shock cognate protein 70 (Hsc70) (Refs 230, 231) and that mild proteasome inhibition led to PrP<sup>TSE</sup> accumulation in the form of large cytoplasmic perinuclear aggresomes (Ref. 230). In the aggresome, PrP<sup>TSE</sup> co-localised with 20S proteasome and ubiquitin, and PrP<sup>TSE</sup> aggresome formation was associated with caspase-8 and -3 activation and apoptosis (Ref. 230), hence confirming previous evidence suggesting caspase-3 activation in affected brain areas of scrapie-infected mice (Refs 201, 232). Later studies provided strong evidence that accumulation of PrP<sup>TSE</sup> in the cytosol inhibits the UPS, and partially explained the molecular mechanisms behind this inhibition. Thus, in N2a cells stably expressing the fluorogenic proteasome reporter substrate Ub<sup>G76V</sup>-GFP, scrapie infection led to increased levels of the reporter protein, while curing cells of infection with the anti-PrP monoclonal antibody ICMS18 normalised substrate degradation (Ref. 231). Aggregated  $\beta$ -sheet rich PrP ( $\beta$ -PrP) and small PrP<sup>TSE</sup> oligomers specifically inhibited the proteolytic activities, namely chymotrypsin-like and caspase-like activities, of the 26S proteasome beta subunits as a result of decreased gate opening of the 20S particle, but not via dissociation of the 26S proteasome (Ref. 231). Supporting evidence was obtained *in vivo* with transgenic mice expressing the Ub<sup>G76V</sup>-GFP reporter protein. These mice displayed diminished capacity to degrade the reporter protein, which accumulated as aggregated deposits in the cytosol of neurons in the most affected brain regions during scrapie infection (Ref. 231). Importantly, the recent identification of changes in the relative levels of heat shock protein HSPA5 and chaperonin TCP1 in mice infected with RML, is consistent with the involvement of the UPS in prion neurodegeneration (Ref. 178).

In sharp contrast are findings in mouse models of inherited prion disorders (Ref. 233). Double-Tg mice, co-expressing PrP with a nine-octapeptide-repeat insertional mutation (PG14) and the Ub<sup>G76V</sup>-GFP reporter, displayed no evidence of UPS impairment despite

showing cytosolic PrP accumulation. Pharmacological induction of cytosolic PrP accumulation in hippocampal neurons from Ub<sup>G76V</sup>-GFP and wild type mice was not accompanied by changes in proteasome activity. No difference in disease onset and progression was reported as compared to Tg(PG14) mice that do not express Ub<sup>G76V</sup>-GFP. In addition, proteasome activity was not altered in primary CGN isolated from Tg mice expressing the D177N/128V mutation (murine PrP numbering). A similar mutation in humans is associated with fCJD (Ref. 233).

A genetic basis for the role of the UPS in TSEs was found in a recent study showing association between the haplotypes of the *HECTD2* gene, encoding an E3 ubiquitin ligase, and the susceptibility to sCJD, vCJD and kuru in humans, and the length of the incubation time in mice (Ref. 234). Genotype-associated differential expression of *Hectd2* mRNA was also reported in mouse brains and human lymphocytes, with a significant up-regulation of *Hectd2* expression in mice during the course of prion disease (Ref. 234).

While different mechanisms of neurodegeneration may occur in inherited TSEs and prion forms acquired by infection (Refs 235, 236), further research is needed to reconcile the existing discrepancies. In this line, of particular relevance are the findings derived from the proteomic analysis of RML-infected wild-type and Tg44 mice described above (Ref. 178).

*Nicotine adenine dinucleotide (NAD<sup>+</sup>) starvation.* Following up on the empirical observation that changes in the composition of culture medium delayed neuroblastoma cell death after exposure to TPrP, Zhou and colleagues identified NAD<sup>+</sup> starvation as a novel mechanism of neuronal death in protein misfolding neurodegenerative diseases (Fig. 2, solid grey line) (Ref. 191). NAD<sup>+</sup> levels were significantly reduced in neuroblastoma cells three days following TPrP treatment, but not after exposure to nontoxic monomeric PrP<sup>C</sup>. Such an effect was normalised by the addition of NAD<sup>+</sup> or the NAD<sup>+</sup> precursor nicotinamide. Since NAD<sup>+</sup> is a co-enzyme critical for energy production, redox homeostasis, Ca<sup>2+</sup> signalling, and post-translational modifications, the authors underlined NAD<sup>+</sup> starvation and related metabolic failure as the most likely primary cause of neuronal death. NAD<sup>+</sup> levels were elevated in astrocytes, a finding consistent with the previously observed resistance to TPrP toxicity (Ref. 170). Because reactive astroglyosis is a well defined characteristic of prion pathology with astrocytes replacing dying neurons (Ref. 237), it was suggested that elevated astrocytic NAD<sup>+</sup> levels compensated the reduced neuronal NAD<sup>+</sup> quantities, therefore explaining the unchanged levels of total NAD<sup>+</sup> observed in prion afflicted mouse brains (Ref. 191). Further investigations demonstrated that NAD<sup>+</sup> depletion triggered autophagy activation, probably as a result of limited ATP supply, suggesting that autophagy,

similarly to apoptosis, is a secondary mechanisms of neurodegeneration in prion diseases (Ref. 191).

### Therapeutic approaches

At present, no efficient therapies are available to treat prion diseases (Ref. 238). Therapies can be directed to various targets: PrP<sup>C</sup>, PrP<sup>TSE</sup>, PrP<sup>C</sup> to PrP<sup>TSE</sup> conversion, and specific neurodegenerative pathways. To date, most approaches are based on inhibiting PrP<sup>TSE</sup> accumulation. Although targeting PrP<sup>TSE</sup> is a potentially powerful approach, it has some drawbacks. Since no pre-symptomatic tests are currently available to diagnose prion diseases, these treatments are administered during the clinical phase, after PrP<sup>TSE</sup> accumulation has long been established. However, it is well known now that loss of synapses and dendrites followed by synaptic dysfunction are early events in prion neurodegeneration preceding PrP<sup>TSE</sup> accumulation. Therefore, therapies aiming at removing PrP<sup>TSE</sup> aggregates may provide little benefit to clinically sick patients. Moreover, in light of the prevalent view that oligomeric rather than fibrillar accumulations of PrP<sup>TSE</sup> are the neurotoxic species, breaking up PrP<sup>TSE</sup> aggregates may exacerbate or even prolong the disease.

Alternatively, targeting PrP<sup>C</sup> offers a number of advantages. PrP-null mice, goats, and cattle are refractory to prion infection and live normal lives (Refs 48, 50, 51, 52, 53, 63). Likewise, knocking out neuronal PrP<sup>C</sup> half-way through the incubation period prevents the development of prion disease (Ref. 160). Unfortunately, transgenic strategies to eliminate PrP<sup>C</sup> expression are far from being applicable to human therapy at the present time. Instead, it is possible to screen for, or design, pharmaceutical inhibitors of PrP<sup>C</sup> biogenesis. Notwithstanding, uncertainty arises from zebrafish studies highlighting the importance of PrP<sup>C</sup> in cell-to-cell adhesion, and until the observed phenotypes in zebrafish and mammalian knock-out models are further explained, approaches targeting PrP<sup>C</sup> expression should be considered with caution. In addition, PrP<sup>C</sup> has been shown to negatively regulate BACE-1 ( $\beta$ -site APP cleaving enzyme 1), and increased levels of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> have been found in PrP null mice, in mice harboring mutations in the *Prnp* gene associated with some forms of fCJD and GSS, as well as in mice infected with various strains of PrP<sup>TSE</sup> (Ref. 122). Thus, removal of PrP<sup>C</sup> or interfering with its physiological function may contribute to the development of AD.

In light of recent observations (Ref. 222), targeting the UPR appears as a very attractive approach. Reduction of eIF2 $\alpha$ -P levels by lentiviral-induced overexpression of an eIF2 $\alpha$ -P specific phosphatase, rescued the pathologic phenotype associated with sustained translational repression during ER stress, and significantly increased mouse survival in scrapie infected Tg37 transgenic mice (Ref. 222). Moreover, pharmacological modification of the UPR by selectively inhibiting PERK phosphorylation and activity upstream of

eIF2 $\alpha$ , prevented translational repression with subsequent neuroprotection. The concomitant pancreatic toxicity observed in treated mice, which led to significant body weight loss and a mild increase of glucose levels in blood, questioned the clinical application of UPR inhibitors and stimulated the search for new compounds with reduced toxicity (Ref. 239). Additional studies from the same group identified the small molecule ISRIB (integrated stress response inhibitor), which prevents translation inhibition downstream of eIF2 $\alpha$ -P, as a good drug candidate. Daily intraperitoneal administration of ISRIB to prion infected Tg37<sup>+/-</sup> mice from 7 weeks post infection, showed that partial restoration of global translation rates sufficed to confer neuroprotection in the absence of pancreatic toxicity. Notably, despite significantly increasing mouse survival as compared to untreated controls, ISRIB administration resulted in significant body weight loss similarly to PERK inhibition (Ref. 240). Understanding whether the observed weight loss results from UPR inhibition, it is a side effect of ISRIB treatment, or else it arises as a consequence of persistent prion infection will have important implications in the clinical implementation of this and similar drugs (Ref. 240).

One of the most promising therapies for treating human prion diseases is passive immunisation with anti-PrP antibodies (Refs 241, 242). This strategy has been extended to several neurodegenerative diseases, including TSEs, encouraged by the observed reduction in amyloid plaque burden in a transgenic mouse model of AD treated with A $\beta$ -directed antibodies (Ref. 243). Additionally, several in vitro studies demonstrate the feasibility of this approach to cure or prevent cellular infection with several strains of prions (Refs 244, 245). Importantly, a recent in vivo study demonstrated PrP<sup>TSE</sup> clearance, and increased survival, after passive immunisation with anti-PrP monoclonal antibodies administered before disease onset (Ref. 246). These findings indicate that therapeutic treatments aiming at blocking the conversion mechanism leading to PrP<sup>TSE</sup> generation are good approaches to treating prion diseases. Nonetheless, the practical application of these therapies depends upon the development of reliable diagnostic tests to allow early antibody administration.

Active immunisation has been evaluated in a number of studies with highly encouraging outcomes [reviewed in (Refs 241, 242)]. Initial studies with bacterially-expressed recombinant full length PrP<sup>C</sup> and various PrP peptides served to highlight the difficulty in breaking tolerance to a self-protein. Additionally, they stressed the importance of breaking tolerance to raise antibodies interfering with PrP<sup>TSE</sup> replication and propagation, while at the same time, minimising an autoimmune response (Ref. 241). Along these lines, mucosal immunisation using bacterial vectors has been suggested as an ideal means to achieve immunomodulation by inducing a secretory IgA response with limited systemic IgG levels, and minimal autoimmune

inflammatory effects. This approach was validated in vivo when wild-type mice previously immunised with a mouse PrP<sup>C</sup>-expressing attenuated *Salmonella* strain, showed resistance to orally administered prions. These mice were characterised by significant anti-PrP mucosal IgA and systemic anti-PrP IgG response (Ref. 247). Mucosal immunisation to prevent CWD infection has also been attempted. White-tailed deer immunisation with attenuated *Salmonella* expressing deer-PrP<sup>C</sup> resulted in increased titers of anti-PrP IgG and IgM in the plasma, and anti-PrP IgA in saliva and faeces as compared with control deer, showing for the first time the generation of humoral responses against self-PrP in the biological fluids of large cervid animals (Refs 241, 248). Vaccinated deer had a significantly prolonged incubation period than control animals following oral infection with a 100% lethal dose of CWD prions. One deer in the vaccinated group remained free of CWD symptoms and PrP<sup>TSE</sup> for 3 years and 7 months, as determined by immunohistochemical evaluation of tonsil and rectoanal mucosa-associated lymphoid tissue biopsies. Importantly, this animal showed the highest levels of mucosal and systemic immune response (Ref. 248).

This study revealed the feasibility of utilising bacterial vectors to induce mucosal immunisation and prevent prion disease infection, albeit being at an early stage of development. Mucosal immunisation represents an important approach to prevent acquired forms of TSE in humans (vCJD), and interrupt transmission of BSE, scrapie, and CWD in animals in which the gut is the major route of entry. Additionally, monoclonal antibodies produced in vaccinated animals could be isolated and used for passive immunisation, extending the potential clinical application of these approaches to other forms of TSE of different aetiology (Ref. 241).

The recent discovery of NAD<sup>+</sup> starvation as potential cause of neuronal death, led to the therapeutical investigation of NAD<sup>+</sup> replenishment in in vivo models of mouse scrapie. Stereotaxic injections of TPrP in the presence or absence of NAD<sup>+</sup> resulted in TPrP toxicity abrogation in a dose-dependent manner (Ref. 191). When NAD<sup>+</sup> was intra-nasally administered at disease onset, mice infected with RML, showed slower disease progression and weight loss than phosphate-buffered saline (PBS)-treated control mice. A similar result was obtained when RML-infected mice were treated during the clinical phase of the disease. Although no differences in survival were observed between treated and control groups, NAD<sup>+</sup> administration significantly increased motor function in mice (Ref. 191).

### Concluding remarks

Prion diseases are devastating disorders of the CNS. At the present time there is no efficient treatment or cure for these diseases, and therefore, affected individuals die within months after the first clinical symptoms appear. Identification of the neurotoxic molecule(s) and the cellular pathways leading to neurodegeneration

would facilitate the development of new therapies. Likewise, development of early, noninvasive diagnostic tests is extremely important; epidemiologically, to prevent secondary transmissions, and therapeutically, to allow the administration of effective therapies before extensive brain damage takes place. This is of particular relevance in the case of hereditary/genetic forms of TSE.

Understanding the biology underlying prion-mediated neurotoxicity is challenging given the unprecedented nature of the infectious agent. PrP<sup>TSE</sup> replicates by conferring its aberrant conformation onto a cellular protein, PrP<sup>C</sup>. Studies conducted with PrP<sup>-/-</sup> mice indicated that loss of PrP<sup>C</sup> function cannot account for prion neurotoxicity, as these mice show neither a deleterious phenotype nor altered lifespan. Experimental evidence has been accumulated demonstrating the dissociation between PrP<sup>C</sup> to PrP<sup>TSE</sup> conversion with accumulation of the latter and neurotoxicity. While PrP<sup>C</sup> expression is prerequisite for prion neuroinvasion and propagation, its requirement at the neuronal membrane for neurotoxicity is still matter of controversy. Intriguing is the fact that astrocytes are refractory to TPrP toxicity, similarly to findings derived from the post-natal removal of PrP<sup>C</sup> expression highlighted above. These observations suggest that the neurotoxic entity, namely TPrP or PrP<sup>L</sup>, should be generated within neurons to cause toxicity to these cells. Importantly, the confirmation of these hypotheses relies on the successful isolation of these molecules from naturally or experimentally induced TSE; task that remains highly elusive.

Similarly controversial is the nature of the entity responsible for the neurotoxic cascade observed in TSEs. Whether prion-related neurodegeneration is triggered by the gain of toxic function of low molecular weight PrP<sup>TSE</sup> oligomers, the generation of toxic intermediates, or the subversion of PrP<sup>C</sup> biological function remains to be elucidated. But in light of the heterogeneity in clinical and pathological presentation of this diverse group of disorders, it is very likely that distinct mechanisms are involved in TSE forms of different aetiology.

Altogether, the findings described in this review indicate that significant progress has been made in recent years towards the elucidation of prion-induced mechanisms of neurodegeneration and, more importantly, potential treatments to halt these devastating disorders. Further confirmation of these mechanisms can be accomplished by refining the identification of the molecules involved in the cellular pathways activated during neurodegeneration. Genetic and biochemical methods can be utilised to identify more precisely cellular partners interacting with variants of PrP molecules, and may reveal signalling cascades activated in TSEs and other neurodegenerative diseases that culminate in neuronal death. The involvement of identified partners will subsequently need to be validated in *in vitro* (cell cultures, organotypic slices, mini brains) and *in vivo* models of prion disease. This can

be accomplished, for example, by deleting the expression of the identified molecules in cells or in mice and by creating double-knockout mice and evaluating the pathologic process under these conditions. Eventually, based on findings derived from these studies, appropriate early diagnostics and targeted treatments can be developed and implemented in real life to treat humans and animals.

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#### Further Reading, Resources and Contacts

Creutzfeldt – Jakob disease foundation (<http://www.cjdfoundation.org>). This organization, founded by family members of people afflicted with TSEs, provides valuable information for researchers, CJD patients and their families.

National Prion Disease Pathology Surveillance Center (<http://www.cjdsurveillance.com>). Established in 1997, the surveillance centre monitors the possible occurrence of vCJD in the USA, establishes the diagnosis and precise type of prion diseases, informs caregivers, reports data to the Centers for Disease Control and Prevention (CDC) and the Health Departments to monitor prevalence of prion diseases in the USA, investigates possibly acquired cases, and stores tissues for future research studies.

The National CJD Research and Surveillance Unit (<http://www.cjd.ed.ac.uk>). CJD surveillance in the United Kingdom (UK) was initiated in 1990. This unit has two principal, inter-related functions: CJD surveillance in the UK and research into prion disease and related problems. They operate in close collaboration with the UK Health Departments, the National Blood Authorities, the Health Protection Agency (HPA) and Health Protection Scotland (HPS), as well as local public health teams.

World Organization for Animal Health (OIE) (<http://www.oie.int/en/animal-health-in-the-world/bse-specific-data/>). Established in the eighties, this organization dedicates a web page to the BSE situation in the world.

WHO manual for surveillance of human transmissible spongiform encephalopathies including variant Creutzfeldt-Jakob disease (<http://whqlibdoc.who.int/publications/2003/9241545887.pdf>)

\*Corresponding author:

Paula Saá,  
Scientific Affairs, Holland Laboratory,  
American Red Cross,  
Rockville, MD, USA.  
E-mail: [Paula.Saa@redcross.org](mailto:Paula.Saa@redcross.org)