RATIONAL TARGETING FOR PRION THERAPEUTICS

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Abstract | Prions — pathogens that are lethal to humans and other animals — are thought to be conformational isomers of the cellular prion protein. Their unique biology, and the potential for a wider pathobiological significance of prion-like mechanisms, has motivated much research into understanding prion neurodegeneration. Moreover, concerns that extensive dietary exposure to bovine spongiform encephalopathy (BSE) prions might have infected many individuals — who might eventually develop its human counterpart, variant Creutzfeldt–Jakob disease (vCJD) — has focused much interest on therapeutics. The challenge of interrupting this aggressive, diffuse and uniformly fatal neurodegenerative process is daunting. However, the recent finding that the onset of clinical disease in established neuroinvasive prion infection in a mouse model can be halted and early pathology reversed is a source for considerable optimism. A therapeutic focus on the cellular prion protein, rather than prions themselves, which might not be directly neurotoxic, is suggested.

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PRION diseases, or TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES, are fatal neurodegenerative conditions that affect humans and other animals, and are transmissible within or between mammalian species by inoculation or ingestion. The human prion diseases, traditionally classified into Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease and kuru, have three distinct aetiologies: they might be autosomal dominantly inherited conditions; acquired from exposure to prions; or they might arise sporadically[1] (TABLE 1). Animal prion diseases include scrapie, a common disease of sheep and goats, transmissible mink encephalopathy, chronic wasting disease of deer and elk, and some more recently recognized diseases of captive wild and domestic cats and exotic ungulates[1]. These last forms are caused by the same prion strain that is seen in bovine spongiform encephalopathy (BSE). BSE prions caused a massive epidemic among the UK herd, with a total epidemic size of infected animals estimated at around two million[2], and has now been reported in most member states of the European Union, Switzerland, the United States, Canada and Japan.

The recognition in 1996 of a novel human prion disease, variant CJD (vCJD)[3], and the experimental confirmation that it is caused by BSE-like prions[4–6], has led to fears that a human epidemic will result, as the majority of the UK population was potentially exposed. Fortunately, the number of recognized cases of vCJD has been relatively small (~150) so far, but the number of infected individuals is unknown. Incubation periods when the disease is transmitted between humans, as evidenced by kuru, are known to span decades, and cross-species transmission is invariably associated with a considerable prolongation of incubation periods — the so-called 'species-barrier' effect[7]. Recent mathematical modelling has indicated that the total epidemic might be small[8], but key uncertainties, notably with respect to important genetic effects on the incubation period[9] and the results of anonymous screens for prion infection in archival tissue[10], indicate the need for caution. Also, such models cannot be used to estimate the number of infected individuals, which is most relevant for assessing risks of secondary transmission from blood transfusions and contaminated surgical instruments. It is therefore prudent to assume that most

Table 1 | Classification of the human prion diseases

Aetiology	Phenotype	Frequency
Sporadic		
Apparently random distribution with an annual incidence of 1–2 per million worldwide	Sporadic CJD: multiple distinct prion strain types associated with distinct clinicopathological phenotypes; rarely associated with sporadic fatal insomnia	85%
Inherited		
Autosomal dominant with high penetrance; all are associated with *PRNP*-coding mutations	Highly variable: more than 30 mutations identified; includes GSS disease, familial CJD and fatal familial insomnia phenotypes	~10–15%
Acquired		
Iatrogenic exposure to human prions from medical contact with human cadaveric-derived pituitary hormones, tissue grafts or contaminated neurosurgical instruments	Iatrogenic CJD: typical CJD phenotype following direct CNS exposure; ataxic onset following peripheral infection	<5% (most patients from USA, UK, France and Japan)
Dietary exposure to human prions through endocannibalism	Kuru	Only in a small area of Papua New Guinea; epidemic in the 1950s, with a gradual decline after the cessation of cannibalism
Environmental exposure (presumed to be dietary) to the BSE prion strain	Variant CJD	Mainly in the UK (total so far ~150), 6 in France, individual patients in several other countries

BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; GSS, Gerstmann–Sträussler–Scheinker; *PRNP*, the gene that encodes the prion protein.

PRION
The transmissible agent that is responsible for prion diseases, which, according to the 'protein-only' hypothesis, lacks an agent-specific nucleic acid genome and is composed principally or entirely of a conformational isomer of cellular prion protein. A term that was originally coined by Prusiner from 'proteinaceous infectious particle'.

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES
(Prion diseases). Transmissible neurodegenerative diseases of mammals that are characterized by neuronal loss, astrocytosis, spongiform vacuolation and accumulation of the disease-associated isoform of prion protein (PrP^{Sc}), sometimes with the formation of amyloid deposits. They are transmissible both within and between species.

PrP^{C}
The normal cellular isoform of mature prion protein. It is a glycosyl phosphatidylinositol (GPI)-anchored cell surface molecule that is highly expressed in neurons, and often associated with raft-like structures in the outer leaflet of the plasma membrane. It consists of a globular, predominantly α-helical, carboxy-terminal domain and an amino-terminal tail that is unstructured in conditions used for solution structure determination.

PrP^{Sc}
The disease-associated isoform of prion protein (PrP), which was originally distinguished from PrP^{C} by its biochemical properties — its relative resistance to proteolysis and detergent insolubility. It is isolated from infected animals as highly aggregated material that appears rich in β-sheet secondary structure. Although it is sometimes used as shorthand for the infectious agent, neither PrP^{Sc} nor the infectious agent itself have yet been clearly defined at the atomical level.

β-PrP
A β-sheet-rich monomeric form of prion protein (PrP) that can be generated from disulphide-reduced PrP in acidic conditions *in vitro*. β-PrP has PrP^{Sc}-like properties and rapidly aggregates and then forms fibrillary structures in physiological salt concentrations.

vCJD cases are yet to occur and might not present clinically for many years[7]. Alternative phenotypes of human BSE infection are also predicted[5,11].

Prion diseases are all associated with the accumulation in the brain of an abnormal, partially protease-resistant, isoform of host-encoded prion protein (PrP). The normal cellular isoform, PrP^{C}, is widely expressed, but is most abundant in the CNS as a glycosylated, glycosylphosphatidylinositol (GPI)-anchored cell surface protein. The disease-related isoform (PrP^{Sc}) is derived from PrP^{C} by a post-translational process that involves conformational change and aggregation. PrP^{C} is rich in α-helical structure, whereas PrP^{Sc}, which is found as insoluble aggregates, seems to be predominantly composed of β-sheet structure. Many studies support the 'protein-only' hypothesis[12,13] — namely that an abnormal PrP isoform is the principal, and possibly the sole, constituent of the transmissible agent or prion. PrP^{Sc} is thought to act as a conformational template, recruiting PrP^{C} to form more PrP^{Sc}. Prion propagation might involve the recruitment of an alternately folded form of PrP^{C}, β-PrP, into PrP^{Sc} aggregates, a process that is thermodynamically driven by intermolecular interactions[14] (FIG. 1).

However, the cause of neuronal death in prion disease remains unclear. The assumption that neurodegeneration follows from direct toxicity of PrP^{Sc} and/or prions[15] (whether or not these are identical) has been increasingly challenged, not least by the recognition of SUB-CLINICAL PRION INFECTION, the state in which animals can have high levels of infectivity without clinical disease. Indeed, several experimental models seem to uncouple prion propagation and neurotoxicity. Most putative therapeutic agents target the accumulation of PrP^{Sc}, but these have shown only modest efficacy in prion-infected animals. At best, they prolong incubation periods, even if given before, or at the at the time of, peripheral infection, but they do not prevent neurodegeneration and death[16]. Indeed, it is now clear that PrP^{Sc} does not directly cause neurotoxicity, but manifests its toxicity only where PrP^{C} is also expressed. Although the precise cause of neuronal cell death remains unclear, the participation of neuronal PrP^{C} is crucial. That prion neurodegeneration could result from the loss of PrP^{C} function — as PrP^{C} is sequestered into aggregated material — seems to be ruled out by recent studies[17]. Therapeutic strategies that deplete neuronal PrP or restrict its recruitment into PrP^{Sc} — rather than targeting PrP^{Sc} itself — now indicate a clear way forward to halt prion neurodegeneration. Here, we review the mechanisms by which prions might cause neurotoxicity and consider therapeutic strategies in the light of these advances and emerging interventional technologies.

Points of intervention

Peripheral infection of laboratory animals with prions is associated with a prolonged, clinically silent, incubation period before CNS invasion and neurological disease can be detected. In natural sheep scrapie and most rodent scrapie models, prion replication is first detectable in the lymphoreticular system (LRS), and CNS invasion is thought to arise by ascending prion spread from infected autonomic nerves that innervate the LRS and visceral tissues. In other animal models, direct infection of peripheral nerves might occur without detectable LRS propagation. In humans, pathogenesis also varies.

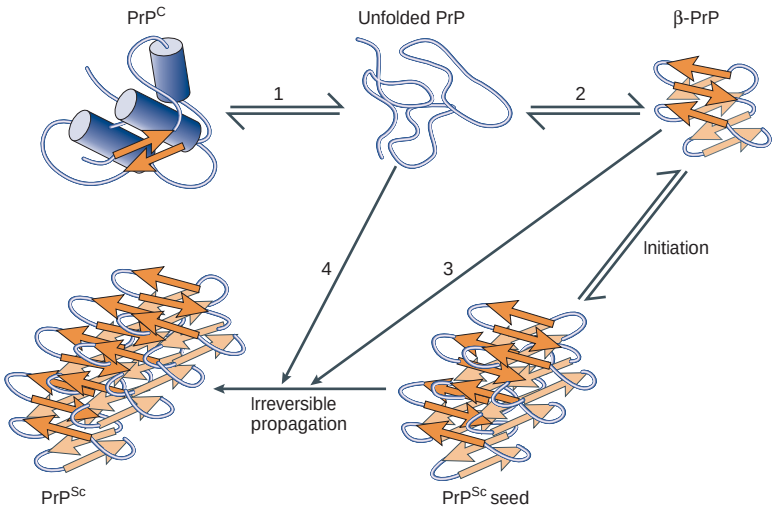

Figure 1 | **A model of prion propagation.** The normal cellular isoform of prion protein, PrP^{C}, is rich in α-helix (blue cylinders) and can be reversibly interconverted (1,2) to a β-sheet-rich (arrows) conformation, β-PrP. Hydrogen–deuterium exchange measurements indicate that adoption of such a radically different fold must proceed through a highly unfolded state[98]. β-PrP has an increased propensity to aggregate, and the formation of a crucial seed size leads to essentially irreversible propagation of the disease-related isoform of the prion protein, PrP^{Sc}, through the recruitment of further β-PrP monomers (3) or unfolded PrP (REF. 1) (4). Subsequent mechanical breakage or cleavage of elongating fibrils would lead to an exponential rise in prion titre. Such a model can accommodate the different aetiologies of human prion diseases (TABLE 1): prion propagation might be initiated by the introduction of a seed (acquired prion disease); by spontaneous seed production as a rare stochastically mediated event that involves wild-type PrP (sporadic prion disease); or as an essentially inevitable event with PrP that contains a pathogenic mutation (inherited prion disease). Following such initiating events, the process of propagation is driven thermodynamically by intermolecular association. Figure modified, with permission, from REF. 1 © (2001) Annual Reviews.

In vCJD, there is uniform and marked LRS infection that involves gut-associated lymphoid tissue, although PrP^{Sc} is not readily detectable in LRS tissues from patients with iatrogenic CJD[18,19]. The aetiology of sporadic CJD is unknown, but might involve *de novo* prion replication, either as a result of somatic mutation of the gene that encodes PrP (*PRNP*) or the spontaneous generation of PrP^{Sc} from wild-type PrP^{C} as a rare stochastic event (TABLE 1). The site of origin of prion propagation in a given patient with sporadic CJD is unknown, and presumably varies, but is probably in the CNS; a similar assumption is made with respect to the inherited prion diseases, in which, as with sporadic CJD, infectivity is largely concentrated in the CNS. Prions are more often detected outside the CNS in patients with sporadic CJD that has longer clinical durations, which might reflect a spread of prions to the periphery.

The peripheral phase of prion propagation offers an opportunity for treatment to eradicate infection before neuroinvasion and might not require a therapeutic agent that can cross the blood–brain barrier. However, the early identification of asymptomatic infected individuals is not yet possible, although at-risk groups are known (for example, those who have been exposed to contaminated pituitary hormones or recipients of blood products from a vCJD-infected donor). Although tonsil biopsy can be used to diagnose vCJD at an early stage, and prions are known to be detectable in LRS tissues preclinically[20], as would be expected from animal pathogenesis studies, this is not suitable for large-scale screening. A diagnostic test that is based on the detection of PrP^{Sc} or specific surrogate markers in the blood remains a key research goal. In the inherited prion diseases, genetic screening can identify presymptomatic affected individuals. There is a real need for developing therapies to target established CNS disease, to prevent neurotoxicity and to salvage damaged neurons. Studies of animal models indicate that not only can disease progression be arrested in principle, but that pathology is reversible, leading to the possibility that early symptoms might be reversible.

Mechanisms of neurotoxicity

The molecular basis of prion neurotoxicity remains an important and controversial subject. Understanding the nature of the neurotoxic species and mechanisms of neuronal damage could guide the development of rational therapies.

There is growing evidence to indicate that PrP^{Sc} itself is not neurotoxic, contrary to the dogma that PrP^{Sc} directly causes neurodegeneration. Although PrP^{Sc} is associated with neuropathological features of prion disease and with infectivity, it does not seem to have direct toxic effects on neurons *in vivo*. *In vitro* studies have, however, indicated that both full-length PrP^{Sc} (REF. 21) and shorter fragments[22] that are derived from PrP^{Sc} are toxic to primary cultured neurons. There are several inherited prion diseases in which PrP^{Sc} is not detected in significant amounts[23–25], and the degree of PrP^{Sc} accumulation in specific brain regions in these cases does not necessarily correlate with clinical features. Inoculation of PrP-null ($Prnp^{0/0}$) mice with prions or PrP^{Sc} has no adverse effects on PrP-null neurons[26–28]. Prion neurotoxicity is confined to PrP-expressing neural tissue that is grafted into $Prnp^{0/0}$ brains, whereas adjacent PrP-null tissue remains perfectly healthy despite accumulation and extensive migration of PrP^{Sc} (REF. 29), indicating that host PrP^{C} is necessary for prion-induced neurotoxicity. Although this is consistent with the protein-only hypothesis of prion propagation, it does not explain the mechanism of neurotoxicity or the role of PrP^{Sc} in this neuronal damage. PrP^{Sc} accumulation can occur without obvious neurotoxic effects, even if PrP^{C} is present to support prion replication throughout the brain[30], or if replication is extra-neuronal[31]. Increasing evidence indicates that there might be sub-clinical forms or 'carrier states' of prion infection, in which high levels of infectivity and PrP^{Sc} are found in animals that do not develop clinically apparent disease during a normal life-span[32,33].

If PrP^{Sc} itself is not directly neurotoxic, is it the depletion of PrP^{C} during prion replication that damages cells? Although the normal function of PrP^{C} is not known, recent evidence indicates that it might be neuroprotective and that aberrantly processed[34,35] or abnormal topological variants of PrP^{C} (REFS 36,37) might be neurotoxic. Although CONDITIONAL KNOCKOUT of PrP showed that its loss of function is not sufficient to cause neurodegeneration (see below), the evidence with respect to aberrant PrP^{C} processing is conflicting.

SUB-CLINICAL PRION INFECTION
Prion diseases are typically associated with prolonged, clinically silent incubation periods before overt neurological disease occurs. However, asymptomatic carrier states of prion infection — sub-clinical infections — have been described in animal models in which animals do not develop clinical prion disease during their lifespan despite high prion titres in the brain.

CONDITIONAL KNOCKOUT
A process by which a gene or its product is knocked out using conditional genetic or recombinant DNA technology. Temporal and spatial features of the knockout are determined by promoter and other elements that control the expression of the protein that is expressed to delete it, such as the phage P1 enzyme Cre recombinase.

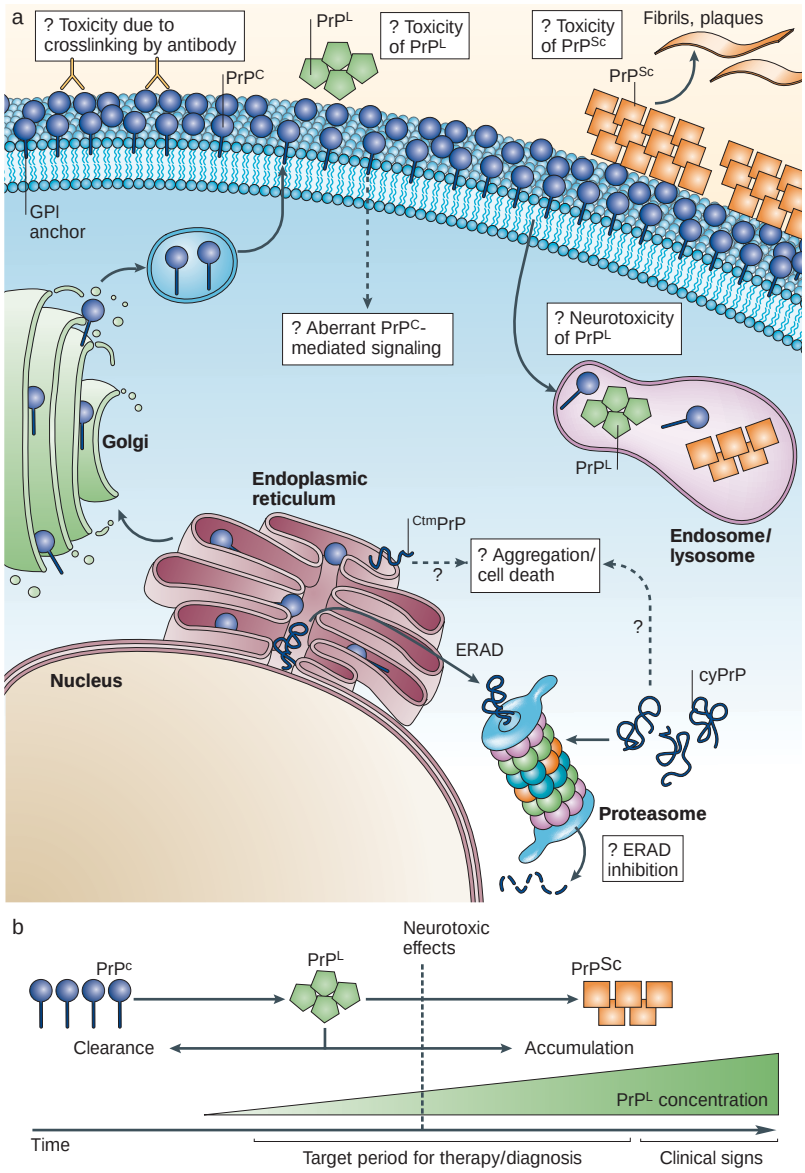

Figure 2 | **Hypothetical mechanisms of prion neurotoxicity a** | A model of potential neurotoxic mechanisms in prion disease. The normal cellular isoform of prion protein, PrP^C (dark blue circles), is synthesized, folded and glycosylated in the endoplasmic reticulum (ER), where its glycosyl phosphatidylinositol (GPI) anchor is added, before further modification in the Golgi apparatus. Mature PrP^C translocates to the outer leaflet of the plasma membrane, where it trafficks in and out of lipid rafts and where it is in proximity to raft-associated signalling molecules. During prion infection, crosslinking of PrP^C could potentially activate cell death or aberrant signalling mechanisms, as seen when extensive neuronal apoptosis occurs after the stereotactic administration of PrP-crosslinking antibody[55]. Other investigators have suggested that sequestration of PrP^C during conversion to the disease-related isoform of the prion protein, PrP^{Sc} (orange squares), contributes to loss of physiological signalling mechanisms, and consequently to the development of neurotoxicity[59–61]. Conversion of PrP^C to PrP^{Sc} could occur through the hypothetical toxic intermediate PrP^L (PrP lethal; green pentagons), which might exist as oligomers, with direct neurotoxic effects both on the cell surface and within late endosomes/lysosomes, where conversion is thought to occur. The rate of PrP^L accumulation would determine neuronal viability. It has been suggested that misfolded and aberrantly processed PrP (cyPrP and ^{Ctm}PrP, respectively) (dark blue coils), which would normally be degraded by the proteasomes through the ER-associated degradation (ERAD) pathway, aggregate in the cytoplasm and cause cell death[76]. Putative proteasomal inhibition or malfunction during prion disease would contribute to this route of toxicity[36,37]. Non-secreted misfolded PrP^c (^{Ctm}PrP) remains in the ER and might also be cytotoxic. **b** | Formation of a hypothetical toxic intermediate (PrP^L), possibly an oligomer, during the conversion of PrP^C to PrP^{Sc} (REF. 33). It is proposed that low levels of PrP^L might be tolerated by the cell. However, when its rate of production exceeds natural clearance, a neurotoxic threshold level of PrP^L is eventually reached and cell death begins, leading to the onset of the clinical syndrome. Part **b** modified, with permission, from REF. 33 © (2003) Elsevier Science.

PrP^C-mediated signalling. PrP-knockout mice are healthy and viable with only minor defects in biochemical[38] and neurophysiological functions[17,39–42]. There is no evidence of neurodegeneration in the absence of PrP^C, regardless of whether the gene is constitutively[43,44] or conditionally[17] deleted. However, PrP-deficient neurons are more susceptible to oxidative stress *in vitro*[45], and show an increased tendency to undergo apoptosis in a serum-free medium — an effect that can be rescued by expression of either B-cell lymphoma protein 2 (BCL2) or PrP (REF. 46). PrP^C can be neuroprotective against various insults *in vitro* and in animal models[47–49]. For example, expression of amino (N)-terminally truncated PrP molecules (ΔPrP32-121 or ΔPrP32-134) in PrP^C-knockout mice results in the extensive degeneration and death of cerebellar granule cells by 6–8 weeks of age, which can be rescued by co-expression of wild-type PrP^C (REF. 50). Intriguingly, the Doppel protein (DPL), which has a similar structure to ΔPrP, causes a similar cerebellar effect when ectopically expressed in the brain[50,51]. The severity of neurotoxicity correlates with the level of DPL expression[52] and can be rescued by PrP^C expression[53], indicating that PrP^C and DPL/ΔPrP might compete for a common receptor or ligand that transduces neuroprotective signals when bound to PrP^C but not when bound to DPL or ΔPrP (REFS 50,54).

Injection of antibodies that recognize and crosslink PrP^C into the mouse hippocampus results in rapid and extensive neuronal apoptosis[55], indicating that putative PrP^C signalling pathways might be involved in cell survival and suggesting that oligomeric PrP^{Sc} molecules or other intermediate species (see below) might crosslink PrP^C and cause neurodegeneration in a similar way.

How PrP^C might act as a signal transducer is not clear. PrP^C is a raft-associated GPI-anchored protein[56]. Antibodies that crosslink such proteins can activate raft-associated signalling molecules[57], but this is well established only for T-cell receptors[58]. However, crosslinking of PrP^C by antibodies[59] or peptides that bind to PrP[49,60] can activate non-receptor tyrosine kinases and other signalling pathways, and the activation is associated with neurite outgrowth, cell survival and protection against apoptosis[49]. Several putative PrP^C-binding ligands have been identified that might be involved in signal transduction[61–65].

PrP^C trafficking. Abnormal topology or altered trafficking of PrP^C might also underlie toxicity, if such aberrantly processed PrP^C in the cell has toxic effects. After processing in the endoplasmic reticulum (ER) and Golgi apparatus, where it is glycosylated, mature PrP^C is attached to the cell surface by a GPI anchor at its carboxy (C) terminus[66]. Studies in cultured cells show that after reaching the plasma membrane PrP^C is recycled to the interior of the cell[67], at least in part, through clathrin-coated pits[68] and is degraded with a half-life of approximately 6 hours[69,70]. The half life of the protein *in vivo* is unknown.

Further studies of the synthesis and trafficking of PrP^C indicate that, after translocation into the ER, PrP^C can adopt two transmembrane topologies — ^{Ctm}PrP and ^{Ntm}PrP — that have the C or N terminus in the ER lumen, respectively[36]. It has been suggested that ^{Ctm}PrP and

^{Ntm}PrP normally represent a small proportion of the cellular PrP, but that when ^{Ctm}PrP is present in excess it is associated with neurodegeneration[36,37]. Some disease-related PrP mutations predispose ^{Ctm}PrP formation and are associated with low levels of PrP^{Sc} accumulation[37]. The propensity for the formation of these intermediates in prion infection might lead to neurotoxicity without formation of PrP^{Sc}.

PrP^{C} that is aberrantly located or accumulating in the cytoplasm might also be toxic. Protein synthesis in the ER has mechanisms of quality control: misfolded proteins are directed to proteasomes in the cytoplasm for destruction by ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION (ERAD)[71]. ERAD degradation of PrP has been shown for several forms of misfolded and mutant PrP[72–75], but also for native PrP^{C} when inhibitors of proteasomes are used[34,35,74]. Normally, PrP^{C} in the cytosol would be rapidly degraded, but in some cases proteasome inhibition results in aggregation of PrP^{C}, with the acquisition of partial resistance to proteases[74–76] and the capacity for self-replication[76] of these cytosolic PrP^{C} aggregates. Indeed, Ma and co-workers suggest that the transfer of newly synthesized PrP^{C} to the cytoplasm by ERAD could be the mechanism by which PrP^{C} becomes neurotoxic and acquires self-propagating properties that might underlie prion infectivity[35,76]. Although cytoplasmic aggregates of PrP^{C} themselves seem to have no neurotoxic effects[34,74], expression of cyPrP — PrP that lacks signal sequences and the GPI anchor — in the cytoplasm is neurotoxic both *in vitro* and *in vivo*[35].

However, there is much debate as to the role that ERAD naturally has in PrP trafficking, and the toxicity of cytoplasmic PrP. Other workers have found evidence that naturally occurring PrP^{C} in the cytoplasm still has its leader peptide but no GPI anchor, indicating that cytosolic PrP^{C} never enters the ER[77] and is therefore not susceptible to ERAD. Furthermore, they found that neither ^{Ctm}PrP nor cytosolic PrP increased in scrapie-infected cultured cells or rodent brains during prion infection[78].

So, the role of ERAD in the intracellular trafficking of PrP and the toxicity of cytoplasmic PrP are controversial. The presence of PrP^{C} in the cytoplasm might be a normal feature of PrP^{C} metabolism, either by retro-translocation or by direct transfer from the ribosomes without entering the ER. In both cases, degradation by the proteasomal pathways would be the normal outcome. Functional impairment of this pathway — due to stress, ageing or primary PrP aggregation[79] — might result in accumulation of PrP^{C} in the cytoplasm, with neurotoxic effects in some cases.

PrP intermediates. During the conversion of PrP^{C} to PrP^{Sc}, a neurotoxic intermediate molecule, which we have termed 'PrP^{L}' (for PrP lethal), might be formed[30,33] (FIG. 2). Recent evidence from the study of soluble precursors of other amyloidogenic proteins associated with neurodegenerative disorders has shed light on such mechanisms. The amyloidogenic proteins associated with Alzheimer's disease, amyloid β40 (Aβ40) and Aβ42, are particularly toxic to cells when they are in the form of small oligomers at the early stage of peptide aggregation before they form Aβ fibrils[80]. During the process of oligomerization of Aβ, reactive oxygen species are generated[81]. When Aβ oligomers are in contact with the cell membranes, lipid peroxidation occurs, which perturbs membrane functions and results in cell death[82]. Antibodies that recognize these Aβ oligomers can inhibit the cytotoxicity not only of Aβ peptides, but also of other amyloidogenic peptides that are involved in prion diseases, Huntington's disease and Parkinson's disease. This indicates that there might be toxic intermediates that share structural similarities despite different amino acid sequences for the various amyloid oligomers and that these might underlie a common mechanism of pathogenesis in different neurodegenerative disorders[83,84].

Although oligomeric forms of PrP might be different from the infectious form of PrP aggregates, they could be rapidly cleared or sequestered into large PrP^{Sc} aggregates. According to this model, the rate of neurodegeneration would depend on the level of steady-state PrP^{L}, which could explain the uncoupling of prion titre and cytotoxicity, as seen in experimental models[33]. Preventing the conversion of PrP^{C} into PrP^{Sc} should also prevent the generation of PrP^{L}, whereas targeting PrP^{Sc}, the end-product of the conversion process, might increase the level of PrP^{L}.

This is consistent with the recent finding that, in infected mice, depletion of PrP^{C} prevents prion propagation in neurons and abrogates prion neurotoxicity[31] (FIG. 3). PrP^{C} depletion in 12-week-old mice that had been inoculated intracerebrally with prions a week after birth reversed the early spongiform vacuolar changes in the hippocampus and protected the animals from neuronal loss and the development of symptomatic clinical prion disease (FIG. 4). These animals remained free of symptoms of prion disease for more than a year after infection, which is close to their natural lifespan.

In addition to important implications for prion therapeutics, these data also shed light on the mechanisms of prion neurotoxicity. PrP^{C} depletion was confined to neurons, so the production of PrP^{Sc} in non-neuronal cells, especially the glia, continued after the loss of neuronal PrP. This extra-neuronal conversion of PrP^{C} to PrP^{Sc} was not toxic to neurons. By 49 weeks post-infection, the animals had accumulated levels of PrP^{Sc} (and prion titres) as high as those seen in end-stage clinical prion disease in normal mice, but no symptoms or neuronal loss were observed[31], which confirms that PrP^{Sc} (and prions) and their accumulation are not intrinsically toxic (FIG. 5). These data are consistent with studies of other sub-clinical models of prion disease[30], which also contest the direct neurotoxicity of PrP^{Sc}.

Therapeutic strategies

The conversion of PrP^{C} to PrP^{Sc} is central to prion pathogenesis. Despite the uncertainties discussed above about the possible mechanisms of neurotoxicity, prevention of this conversion in neurons can prevent disease progression and reverse early degenerative changes[31]. In addition to the goal of preventing the onset or progression of neurotoxicity, it might also be possible to use neuronal precursor cells for neuronal repair.

ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION (ERAD). A quality control mechanism for protein synthesis in the endoplasmic reticulum (ER). Misfolded proteins are directed by the ER to proteasomes in the cytoplasm for degradation.

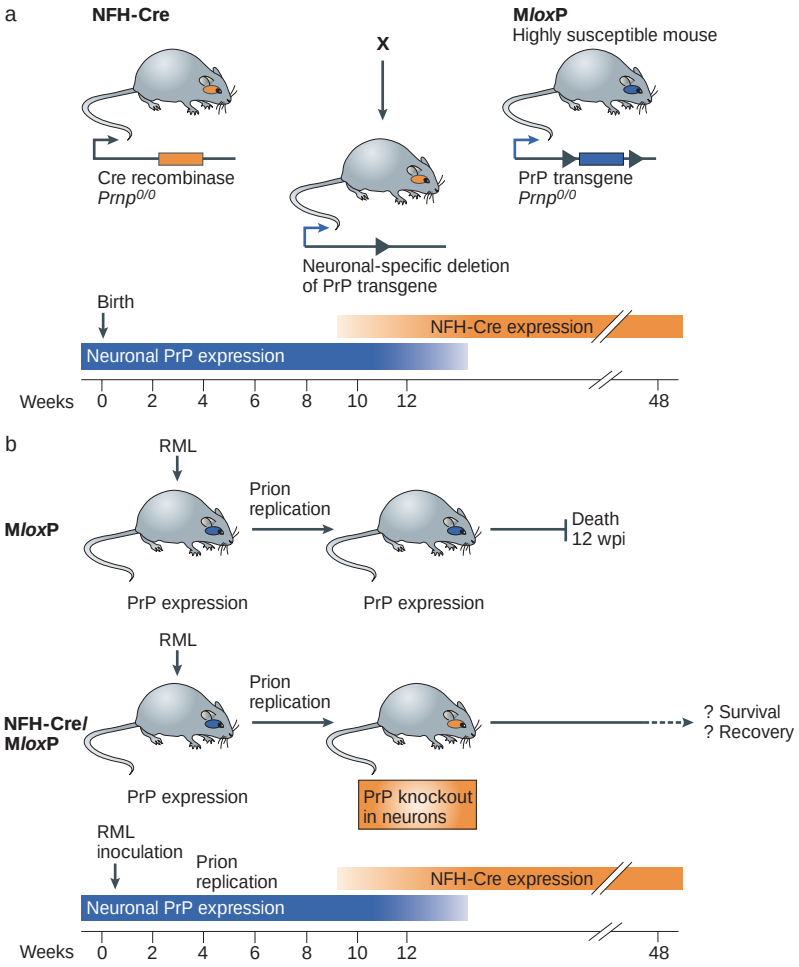

Figure 3 | **Model of late-onset PrP depletion in mice by Cre-mediated recombination.** **a** | In NFH-Cre mice, expression of the phage P1-derived DNA recombinase, Cre, is driven by the promoter of the neurofilament heavy (NFH) chain gene. These mice were crossed with M*lox*P mice, in which the only expression of the normal cellular isoform of prion protein (PrP^C) was from a PrP transgene flanked by M*lox*P sites (mice were generated on a PrP-null (*Prnp*$^{0/0}$) background). The M*lox*P sites (black triangles) are recognition sites for Cre, which excises intervening DNA sequences, and leaves a single *lox*P site after excision. Cre-mediated recombination occurs in neurons of the double-transgenic NFH-Cre/M*lox*P mice at ~10 weeks of age. **b** | Prion challenge of M*lox*P and NFH-Cre/M*lox*P mice. These mice were intracerebrally inoculated with Rocky Mountain Laboratory (RML) prions at 1 week of age, allowing ~9 weeks for prion replication and CNS prion infection to be established before neuronal PrP^C depletion occurred in the double-transgenic NFH-Cre/M*lox*P mice. M*lox*P mice were inoculated at the same time and succumbed to scrapie at 12 weeks post-inoculation (wpi).

Cre RECOMBINASE
Phage P1-derived DNA recombinase that excises DNA sequences that are flanked by *lox*P sequences with the same orientation. It is effective in mammalian cells *in vitro* and *in vivo*.

*lox*P SEQUENCE
A 34-base-pair sequence for recognition by Cre recombinase. One *lox*P site remains after Cre-mediated excision.

Preventing the actions of PrP^{Sc}. Compounds that reduce PrP^{Sc} accumulation in prion-infected cell culture models include polyanionic compounds[85], Congo red[85], amphotericin B[86], porphyrins[87] and phenothiazine derivatives, such as quinacrine[88]. However, few cultured cell lines can support prion propagation, and those that do can be 'cured' relatively easily; also, there is typically no cellular pathology. Such models might, therefore, not be a stringent screen for efficacy. In support of this view, many of these substances have only modest effects on prion incubation periods *in vivo*[89–94] and generally only when administered together with, or soon after, the prion inoculum[92]. Some PrP^{Sc}-binding substances might simply reduce the effective titre of the initial inoculum, rather than abrogating pathogenesis itself. Moreover, many of these drugs are effective only in peripherally infected animals (after experimental intraperitoneal inoculation) before neuroinvasion. Two exceptions are pentosan polysulphate[95] when administered intraventricularly in intracerebrally infected mice, and amphotericin, which can delay clinical onset when administered late in the course of infection[96].

Preventing the conversion process. Possible strategies for preventing conversion include the identification of ligands that can bind to and stabilize PrP^C (which make it less available for conversion), such as the use of antibodies that bind or sequester PrP^C, or methods to downregulate PrP transcription or translation.

Although the precise molecular events involved in the conversion of PrP^C to PrP^{Sc} and the atomic structure of both the infectious and the neurotoxic species remain ill defined, it can be argued that any ligand that selectively stabilizes the PrP^C state should block prion propagation. The propensity of PrP^C to adopt alternative conformations, depending on solution conditions, has been used to argue that there might be an unusually high proportion of partially unfolded forms that are in dynamic equilibrium with the native state[97]. However, the use of hydrogen–deuterium exchange to measure the extent to which regions of PrP^C transiently unfold has shown that the conformation of human PrP^C is not abnormally plastic[98]. All three α-helices show protection factors that are equal to the equilibrium constant for global unfolding of the molecule. Therefore, there are no partially unfolded forms of PrP^C that retain any of the dominant secondary structural motifs[98]. These results show that the conversion of PrP^C to PrP^{Sc} must proceed through a highly unfolded state that retains little organized native structure, and that compounds that can bind to any ordered region of PrP^C should therefore inhibit the conversion pathway. The maintenance of effective brain levels of drugs that reduce prion propagation rates to below those of natural clearance mechanisms could therefore plausibly cure prion infection. High-throughput screening of large compound libraries can be applied to detect such ligands. This approach has been exploited therapeutically for at least two proteins in which mutations or altered conformation result in disease: the central core of tumour protein p53 (TP53, also known as p53) and transthyretin (TTR). Ligand binding maintains stability and function of these proteins, protecting against cancers that arise from destabilizing mutations of the p53 core[99] and reducing amyloid formation from TTR monomers in systemic amyloidosis[100].

Anti-PrP antibodies. Antibodies against certain PrP epitopes inhibit propagation of PrP^{Sc} in cell culture[101–103], and show promise *in vivo*. The antibodies have little or no affinity for native PrP^{Sc}, and might function by binding cell surface PrP^C and reducing its availability for incorporation into propagating prions. Transgenic mice that express anti-PrP μ-chains that are directed against similar epitopes are protected against peripheral (but not central) prion infection, although these mice were not

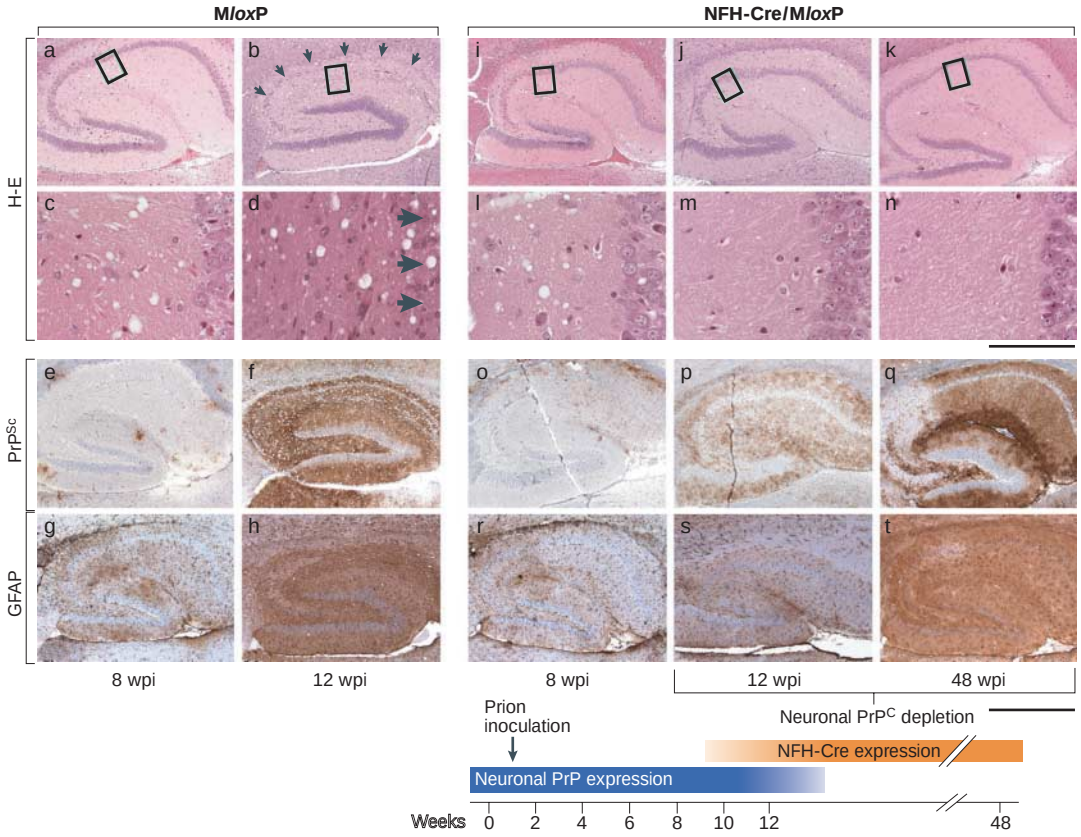

Figure 4 | **PrP^C depletion in neurons of mice with established CNS prion infection reverses early spongiosis and prevents neuronal loss.** In M*lox*P mice, the normal cellular isoform of prion protein (PrP^C) is expressed throughout the infection period. Crossing M*lox*P mice with NFH-Cre mice, in which expression of the phage P1-derived DNA recombinase, Cre, is driven by the promoter of the neurofilament heavy (NFH) chain gene, generates NFH-Cre/M*lox*P mice in which PrP^C is depleted from neurons ~10 weeks post-inoculation (wpi). Fixed hippocampal sections from RML (Rocky Mountain Laboratory) prion-infected M*lox*P and NFH-Cre/M*lox*P mice were stained with haematoxylin-eosin (H-E) or immunostained for glial fibrillary acidic protein (GFAP) for detection of gliosis and deposition of the disease-related isoform of the prion protein (PrP^{Sc}). There is severe loss of CA1–3 neurons (arrows) (**b**,**d**) with shrinkage of the entire hippocampus (**b**) in terminally ill M*lox*P mice, when marked astrocytic gliosis (**h**) and extensive PrP^{Sc} deposition (**f**) are also seen, typical of end-stage pathology in prion infection. There is no neuronal loss in asymptomatic prion-infected mice with Cre-mediated PrP^C depletion (**j**,**k**,**m**,**n**) up to 48 wpi. Early spongiosis was seen in all animals at 10 wpi (**c**,**l**) when both groups expressed PrP^C at the same level throughout the brain. Spongiosis declined at later time points in NFH-Cre/M*lox*P mice (12 and 48 wpi are shown, **m**,**n**) when neuronal PrP^C was eliminated, despite continued extra-neuronal PrP^{Sc} accumulation (**p**,**q**) and gliosis (**s**,**t**). Scale bar = 320 μm, except in panels **c**,**d** and **m**–**p** in which it represents 80 μm. The schematic diagram beneath the histology images represents the time course of neuronal PrP^C expression and its depletion by Cre in inoculated M*lox*P/NFH-Cre mice. Figure modified, with permission, from REF. 31 © (2003) American Association for the Advancement of Science.

immunologically normal[104]. In a clear proof of principle, mice that had been peripherally infected with prions were passively immunized with anti-PrP monoclonal antibodies. PrP^{Sc} levels and prion infectivity in the spleens of scrapie-infected mice were markedly reduced even when antibodies were administered at the point of near-maximal PrP^{Sc} accumulation. Furthermore, treated animals remained healthy more than 300 days after untreated animals had succumbed to the disease[105]. Unsurprisingly, as antibodies do not readily cross the blood–brain barrier, there was no protective effect in intracerebrally infected mice. Nevertheless, humanized anti-PrP monoclonal antibodies might be used for post-exposure prophylaxis of particular at-risk groups. For established clinical disease, such antibodies could, in principle, be given by intracerebroventricular infusion, although effective tissue penetration throughout the CNS might be difficult. However, one study found that the injection of large quantities of anti-PrP antibodies into the CNS caused massive neuronal apoptosis[55], and it will be important to investigate the epitope and dose dependency of this effect.

Active immunization is limited by immune tolerance to PrP, which is widely expressed in the immune system. Approaches to overcome this tolerance are being actively investigated, and studies have reported modest protective effects[106,107].

The rarity of human prion diseases and the consequent difficulty in assessing safety and efficacy means that immunization of healthy individuals against prion diseases might not be practical. However, such a programme might eradicate endemic animal prion diseases such as ovine scrapie. Clinical studies of active immunization for Alzheimer's disease give cause for caution: anti-Aβ40–42 antibodies can have protective or noxious

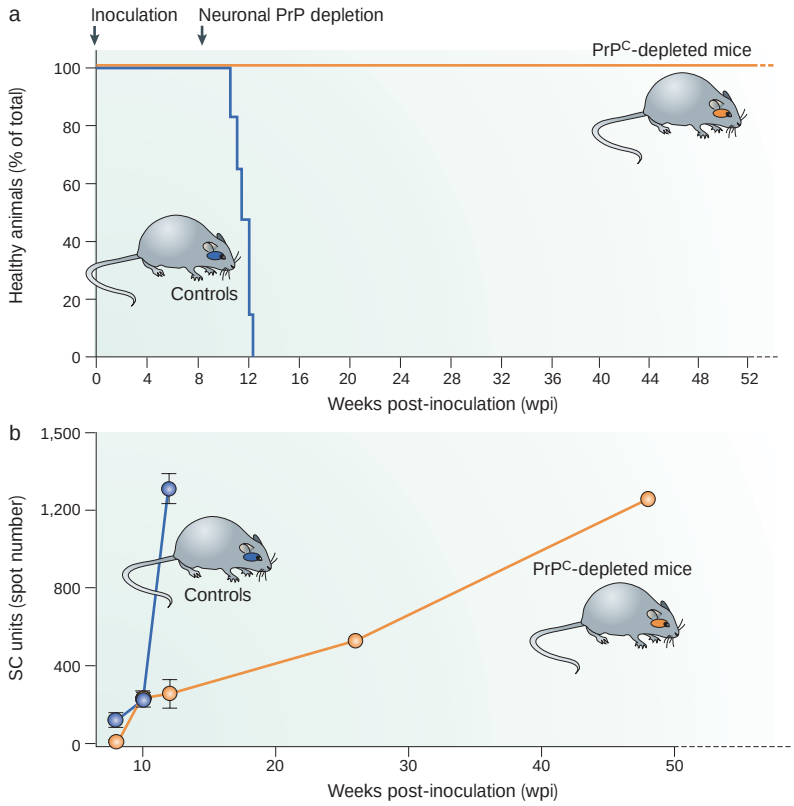

Figure 5 | **Survival of prion-infected mice after PrP^C depletion. a** | In M*lox*P mice (controls), the only expression of the normal cellular isoform of prion protein (PrP^C) was from a PrP transgene flanked by M*lox*P sites (mice were generated on a PrP-null background). The expression of PrP^C occurs throughout the infection period and these animals all succumbed to scrapie prion infection within 12 weeks post-inoculation (wpi). Crossing M*lox*P mice with NFH-Cre mice, in which expression of the phage P1-derived DNA recombinase, Cre, is driven by the promoter of the neurofilament heavy (NFH) chain gene, generates NFH-Cre/M*lox*P mice, in which PrP^C is depleted from neurons ~10 wpi. In contrast to control M*lox*P mice, NFH-Cre/M*lox*P animals remained well without clinical signs of scrapie at 52 wpi. Onset of Cre-mediated PrP^C depletion in NFH-Cre/M*lox*P mice at 10 weeks into the course of infection is indicated. **b** | Prion titre in brain homogenates from M*lox*P mice (controls), which is determined by a scrapie cell (SC) assay[152], increases rapidly until they develop terminal disease and have to be culled[31]. Infectivity in NFH-Cre/M*lox*P mice, with Cre-mediated neuronal PrP^C depletion, lags behind that in controls, but eventually reaches levels seen in terminally ill M*lox*P mice.

effects in cells[108], and a recent anti-Aβ40–42 vaccination trial was halted owing to encephalitic illness in some recipients[109].

Other immunomodulatory approaches to blocking peripheral replication and neuroinvasion in peripherally acquired prion disease have been reported in animal models. B-cell-derived lymphotoxin-α and tumour necrosis factor-α (TNF-α) are involved in the differentiation and maturation of follicular dendritic cells (FDCs), which are required for splenic PrP^{Sc} accumulation and subsequent neuroinvasion[110,111]. Knockout of TNF-α[112] or complement components (which are involved in antigen recognition by FDCs)[113,114] causes an increase in the length of the incubation period. Neutralization of the lymphotoxin-β receptor (LTβ-R) pathway by administration of a soluble LTβ-R-Ig fusion protein blocks FDC maturation and prevents scrapie neuroinvasion if administered soon after oral or intraperitoneal scrapie infection but not after intracerebral infection[115–117].

RNA INTERFERENCE (RNAi). Describes the use of double-stranded RNA to target specific messenger RNAs for degradation, thereby silencing their expression. RNAi is one of several RNA-silencing phenomena that occur in plants, animals and fungi.

Eliminating PrP^C. Constitutive lack of or acquired depletion of PrP^C are tolerated in mice[17,43,44], and PrP^C depletion has therapeutic benefit in prion infection[31]. However, for therapeutic benefit PrP^C knockout must be achievable using extrinsic means. The use of small duplex RNA molecules to silence gene expression in a sequence-specific manner — RNA INTERFERENCE (RNAi) — is a powerful biotechnological tool, with the potential for therapeutic gene silencing. RNAi can suppress gene expression both *in vivo* and *in vitro* in mammalian cells[118,119], including non-dividing cells such as neurons. Incorporation of small duplex RNAs into replication-deficient lentiviruses (and other viral vectors) and the intergration of the virus into neuronal DNA result in stable long-term expression of these RNA molecules and causes RNAi to take place both *in vitro*[120,121] and *in vivo*[122–124]. *In vivo* studies indicate that RNAi could potentially be used to treat polyglutamine diseases[120,121,125], Alzheimer's disease and frontotemporal dementia[126]. *In vivo*, viral vector-mediated RNAi has also been successfully used to treat non-genetic neurological disorders[127], and it has been shown that viruses can be delivered stereotaxically either focally or throughout the brain[128]. RNAi successfully inhibits PrP^C expression in neuroblastoma cells[129] and prevents the accumulation of abnormal PrP^{Sc} in scrapie-infected cells[130]. The prospect of lentivirus-mediated RNA silencing of PrP^C as a therapeutic tool is promising and provides a potential means of extrinsically manipulating PrP gene expression *in vivo*. However, any successful therapeutic delivery system would require effective CNS penetration.

Repair of neuronal damage. Once severe neuronal loss is established, any form of 'rescue' is difficult. Nonetheless, embryonic stem cells[131] and neuronal precursor cells[132,133] can migrate within the brain to sites of injury, including those of neuronal degeneration[134–136], and can differentiate into various neuronal cell types. Such cells would need to be *Prnp* null, unless prion infection could be eradicated. Astrocytes, macrophages[137] and neural precursors[138] can all be transduced for viral delivery and act as vector-producing cells *in vivo*, potentially serving as biological pumps for the delivery of therapeutic molecules such as growth factors[139].

Enhancement of natural prion clearance. Prions are thought to be resistant to proteolytic degradation: however, there is evidence for relatively efficient natural clearance mechanisms. Although PrP^{Sc} is initially defined according to its partial resistance to proteinase K, this is a relative resistance. Mice that lack PrP (*Prnp$^{0/0}$*) cannot replicate prion infectivity[26], and after intracerebral prion inoculation the initial high prion titre (8.5 log LD_{50} units per ml — the dose that kills 50% of inoculated animals) is reduced to below detection limits by natural mechanisms within 2 weeks. In scrapie-infected N2a (ScN2a) cells, PrP^{Sc} is cleared 3 days after transient treatment with monoclonal antibodies against PrP(REF. 101). Although these clearance mechanisms remain to be characterized, enhancement of prion clearance would also represent a rational therapeutic approach.

Other approaches. It is clear from quantitative trait locus linkage studies in mice that several other genetic loci in addition to the PrP gene locus have a significant effect on the incubation period[9,140,141]. Long-term survivors of the epidemic of human prion disease kuru are predominantly heterozygous at the polymorphic codon 129 of *PRNP*[142], a powerful protective factor against prion disease[143,144]. Individuals who have been exposed to prions but are currently unaffected might also have other protective alleles. Conversely, those individuals who have developed vCJD so far might have short incubation alleles at loci that are associated with incubation periods[7]. Efforts are underway in several laboratories to clone these genes, and their identification might open new avenues to delay or prevent disease onset.

Anticipating drug resistance

Prion strains show considerable diversity and are associated with distinct PrP^{Sc} types, which differ in their conformation[4,145–147] and glycosylation[4]. At least four types of PrP^{Sc} are described in sporadic and acquired CJD[4,148]. Strain switching or mutations have been well documented using both biological and molecular strain-typing methods[4,5,149,150], and it seems that more than one identifiable strain might propagate in the same host. BSE prions induce the replication of two distinct prion strains in transgenic mice that express only human PrP[11]. The possibility of the emergence of drug-resistant strains, by the selection of a minor or sub-strain of prion, against agents that target PrP^{Sc} can therefore be anticipated. However, according to the protein-only hypothesis, targeting native PrP^{C} should block replication of all prion strains.

Human therapeutic studies

Many drugs have been given to small numbers of patients with prion disease over several years. With few exceptions[151], reports have been largely anecdotal. There is no clear evidence of efficacy of any agent, and controlled clinical trials are urgently needed. Such trials will be challenging for several reasons. The diseases are rare, and CJD is rapidly progressive and invariably fatal, which might make randomization to placebo unacceptable. Overall patterns of disease are, however, extremely variable; clinical durations can vary from several weeks to more than 2 years in sporadic CJD and more than 20 years in some inherited forms[1]. As 'first generation' treatments for prion disease are likely, at best, to have only a modest effect on disease progression, even survival duration as an outcome measure requires the study of large numbers of patients to clearly examine efficacy. There is a lack of detailed systematic natural history studies of disease progression and an absence of biological markers to study disease activity and response to therapeutic intervention. An added complication is caused by the high profile of these diseases. Reports of putative prion therapeutics are frequently followed by optimistic headline coverage, which, understandably, leads to requests by patients for immediate access and unrealistic expectations of efficacy, not least in patients with advanced neurodegeneration. When the drug fails to have a marked effect, there might then be loss of interest in participation in controlled trials — which necessarily take considerable time to be organized, peer reviewed and ethically approved — to assess whether the drug actually has a worthwhile therapeutic effect. In the United Kingdom, at the request of the government's Chief Medical Officer, a clinical trial protocol and infrastructure has been developed to rigorously assess the drug quinacrine (see online links box)[88] and to provide a framework for the assessment of new therapeutics as they become available. A formal consultation with patients' representatives was carried out to assist the development of a protocol that would be acceptable to most patients and their families (see online links box). Clearly, advances in the early diagnosis of prion infection are urgently needed to allow any experimental therapies to be trialled before extensive neuronal loss has occurred.

Daunting challenge or cautious optimism?

Effective treatment of neurodegenerative disease is one of the principal challenges that currently faces biomedical research. The recent focus on understanding prion neurodegeneration following the BSE epidemic and recognition of its risk to public health — added to the intrinsic interest in the unique pathobiology of prion diseases — has lead to these diseases being among the best understood causes of neurodegeneration. Our understanding of the molecular basis of both prion propagation and prion-related neurotoxicity is advancing rapidly, and the development of rational therapeutics to eradicate prion infection seems to be feasible.

It is to be hoped that prion diseases will remain rare. If no epidemic of BSE-related human infection emerges, such advances, and knowledge of the capacity of the CNS to recover following interruption of a neurodegenerative process, might potentially have far wider value. This improved understanding might be particularly useful for the treatment of other more common neurodegenerative diseases that are associated with the aggregation of misfolded proteins, such as Alzheimer's disease and Parkinson's disease. Conversely, if extensive human infection with BSE prions emerges in the United Kingdom and other countries, and silent secondary transmission is efficient, the failure to have developed such a treatment for this, albeit inadvertently, man-made disease during the window of opportunity that is provided by the remarkable latency of these infections might not be forgiven.

Advances in diagnostics, and in particular the means to identify pre- or sub-clinically infected individuals before neuroinvasion, could then disrupt any emerging human BSE epidemic as well as allow at-risk groups with known significant exposure to be targeted. In patients with established neuroinvasive disease, early diagnosis will be vital to allow intervention before extensive neuronal loss has occurred. However, the animal models discussed here allow at least cautious optimism that some early pathology might be reversible. The detection of vCJD prions by tonsillar biopsy[18] allows early diagnosis in symptomatic individuals with suspected vCJD. In addition to small molecule ligands for PrP^{C}, anti-PrP

antibodies and interference with PrP expression seem to be realistic candidates for anti-prion therapeutics, by depleting the substrate for neuronal prion propagation. However, the unpredictable effects of antibodies for PrP^C (REF. 55) and in therapeutic immunization against Alzheimer's disease are examples of the fine balance between protective and harmful effects[108] of interventions. Further definition of the normal physiological role of PrP^C might help to refine interventional strategies, and the precise characterization of the neurotoxic species and the pathogenic cascades involved in prion-induced neuronal death should clarify both these issues and also allow other targeted approaches to treatment. Nonetheless, the reversal of spongiform change and the long-term neuronal viability of prion-infected mice that are on the cusp of neurodegeneration have brought the prospect that at least partial recovery from even symptomatic prion disease is now a real possibility.

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Competing interests statement

The authors delare competing financial interests: see Web version for details.

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FURTHER INFORMATION

Current Controlled Trials — Quinacrine for human prion disease: http://www.controlled-trials.com/isrctn/trial/PRION/0/06722585.html

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