Review

Pathogenesis of prion diseases: a progress report

A Aguzzi*,1 and FL Heppner1

1 Institute of Neuropathology, Department of Pathology, Schmelzbergstrasse. 12, University Hospital Zurich, CH-8091 Zurich, Switzerland
* Corresponding author: A Aguzzi, Institute of Neuropathology, Schmelzbergstrasse 12, University Hospital, CH-8091 Zürich. Tel: +41 1 255 2107; Fax: +41 1 255 4402; E-mail: adriano@pathol.unizh.ch

Abstract

Almost 20 years have passed since Stanley Prusiner proposed that the agent causing transmissible spongiform encephalopathies consists exclusively of a protein and termed it prion. A mixed balance can be drawn from the enormous research efforts that have gone into prion research during this time. On the negative side, the protein-only hypothesis has not been conclusively proven yet. On the positive side, our understanding of spongiform encephalopathies has experienced tremendous advances, mostly through human genetics, mouse transgenetics, and biophysical methods. Perhaps the most astonishing development is the realization that many human neurodegenerative diseases for which transmissibility has been more or less stringently excluded, may follow pathogenetic principles similar to those of prion diseases. Also, the hypothesis that prion-like phenomena may underlie certain non-genetic traits observed in yeast has resulted in the surprising recognition that the instructional self-propagating changes in protein conformation may be much more prevalent in nature than previously thought. The latter developments have been astonishingly successful, and one could now argue that the prion principle is much more solidly established in yeast than in mammals. *Cell Death and Differentiation* (2000) **7**, 889–902.

Keywords: prion protein; PrP^{Sc}; PrP^C; transmissible spongiform encephalopathies; scrapie; protein-only hypothesis

Abbreviations: PrP^C, cellular prion protein; PrP^{Sc}, scrapie-associated prion protein; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; nvCJD, new variant form of Creutzfeldt-Jakob disease; TSE, transmissible spongiform encephalopathy; Dpl, Doppel; MBM, meat and bone meal; GSS, Gerstmann-Sträussler-Scheinker; ^{CTM}PrP, transmembrane form of the prion protein; FDC, follicular dendritic cell; PS, pentosan polysulphate; PcTS, phtalocyanine tetrasulfonate; iPrP13, 13-residue β-sheet breaker peptide; PrD, prion-forming domain

Introduction

The prion was defined in 1992 by Stanley B Prusiner as the infectious agent that causes transmissible spongiform encephalopathies (TSEs), or prion diseases. According to Prusiner, the prion (from *proteinaceous infectious only*) is devoid of informational nucleic acids[1] and consists of an 'infectious' protein that is capable of converting a normal host protein termed PrP^C (*cellular prion protein*), or simply PrP, into a likeness of itself. This hypothesis is now very widely accepted, although critique continues to be voiced mainly because no direct and incontrovertible evidence has been forthcoming yet.[2] In 1982 Prusiner isolated a protease- and heat-resistant protein from the brain of scrapie-infected hamsters.[3] He termed it PrP^{Sc} (*scrapie-associated prion protein*) and proposed that PrP^{Sc} is identical with the infectious agent. The gene which encodes PrP^c, *Prnp*, was identified later by Charles Weissmann at the Institute of Molecular Biology in Zurich[4] in hamsters, and independently by Bruce Chesebro in mice.[5] *Prnp* is active in brain and many other cells of the body in both prion-infected and non-infected animals and, most strikingly, removal of *Prnp* from the mouse genome does not prevent normal development yet leads to complete immunity from scrapie.[6–8]

The only organ system in which severe histopathological damage can be demonstrated as a consequence of infection with prions is the nervous system. This applies to all human TSEs, such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker Syndrome, Kuru and fatal familial insomnia, and to all known prion encephalopathies of animals, as summarized in Table 1. However, it is apparent that prions can colonize organs other than the central and peripheral nervous system, and can be demonstrated in extracerebral compartments, and fibrosis and weight reduction is often seen in spleens of terminally scrapie-sick mice.

While the prototype of all prion diseases, scrapie in sheep, has been known for more than two centuries, bovine spongiform encephalopathy (BSE) had its first recognition in 1986 and developed into an epizootic phenomenon. The emergence of a new variant form of Creutzfeldt-Jakob disease (nvCJD) in young people in the UK has raised the possibility that BSE has spread to humans by dietary exposure.[9–12] Experimental evidence claiming that the agent causing BSE is indistinguishable from the nvCJD agent supports this view,[13] although no ultimate proof has been presented, due to limitations of the analytical assays used for determining the characteristics of prions. Although most cases of human prion diseases are sporadic, about 10% are familial and linked to one of a number of mutations in the gene encoding PrP. It is believed that these mutations allow spontaneous conversion of PrP^C into PrP^{Sc} with a frequency sufficient to cause disease within the lifetime of an individual.[14]

Table 1 The prion diseases

Disease	Host	Mechanism of pathogenesis
iCJD	humans	infection from priorn-contaminated hGH, dura mater grafts, etc.
nvCJD	humans	infection from BSE prions (hyphothesized)
fCJD	humans	germ-line mutations in *PRNP* gene
sCJD	humans	somatic mutation resulting in spontaneous conversion of PrP^C into PrP^{Sc}?
Kuru	fore people	infection through ritualistic cannibalism
GSS	humans	germ-line mutations in *PRNP* gene
FFI	humans	germ-line mutations in *PRNP* gene
FSI	humans	somatic mutation resulting in spontaneous conversion of PrP^C into PrP^{Sc}?
Scrapie	sheep	infection in genetically susceptible sheep
BSE	cattle	infection with prion-contaminated MBM
TME	mink	infection with prions from sheep or cattle
CWD	mule, deer, elk	Unknown
FSE	cats	infection with prion-contaminated bovine tissues or MBM
Exotic ungulate encephalopathy	greater kudu, nyala, oryx	infection with prion-contaminated MBM

iCJD, iatrogenic CJD; nvCJD, new variant CJD; fCJD, familial CJD; sCJD, sporadic CJD; GSS, Gerstmann-Sträussler-Scheinker disease; FFI, fatal familial insomnia; FSI, fatal sporadic insomnia; BSE, bovine spongiform encephalopathy ('mad cow disease'); TME, transmissible mink encephalopathy; CWD, chronic wasting disease; FSE, feline spongiform encephalopathy; hGH, human growth hormone; MBM, meat and bone meal

Prion diseases – a brief history

Although scrapie in sheep has been known for at least 250 years, only with discovery of transmissibility in the 1930s were the unusual properties of the infectious agents recognized. In particular the long incubation time and resistance to conventional sterilization procedures.[15] About 30 years later, Griffith outlined the 'protein-only' hypothesis[16] that has gained widespread recognition and acceptance after Stanley B Prusiner purified the pathological protein PrP^{Sc} in 1982.[3]

When transmission of scrapie in sheep was described, the high incidence of familial Creutzfeldt-Jakob disease (fCJD) in some families had already been recognized. Some 20 years later, transmissibility of Kuru, a disease of New Guinea highlanders provoked through ritualistic cannabalism, and of CJD to apes could be demonstrated.[17,18] Epidemiological studies designed to identify the source of the CJD infection evidenced geographic clusters: Libyan Jews living in Israel developed CJD about 30 times more frequently than other Israelis:[19] while this was first thought to result from consuming lightly cooked brains from scrapie-infected sheep prior to emigration to Israel, it was subsequently shown that the Libyan Jewish patients carry a mutation at codon 200 (E200K) in *PRNP*, the gene encoding PrP.[20]

In 1986, a novel type of TSE was found to affect cows: bovine spongiform encephalopathy (BSE) or 'mad cow disease'.[21] Most likely it has been transmitted to cows through feeding with rendered offal derived from meat and bones of scrapie-infected sheep – and presumably from BSE-infected cows to humans causing new variant CJD (nvCJD).

The 'protein-only' hypothesis

Although the exact physical nature of the transmissible agent is still controversial, a very large body of experimental data supports the 'protein only' hypothesis, first outlined in general terms by Griffith in 1967[16] and enunciated in its updated and detailed form by Prusiner.[3] It proposes that the agent is devoid of nucleic acid and consists solely of an abnormal conformer of the cellular prion protein, PrP^C. Additional evidence is provided by very recent findings demonstrating that mouse PrP expressed in both yeast and neuronal tumor cells can be converted into an abnormal form close to that adopted by naturally occurring mouse prions. Whether these transgenetically produced prions can infect mice under virus-free conditions has not been determined beyond any doubt: some reports have implied such *de novo* generation of prions,[22] but others appear to have yielded different results.[23] If positive, such experiments may fully prove the 'protein-only' hypothesis and convince skeptics still favoring an as-yet-unidentified agent (be it virus, virino, or one of the more exotic microbes which are periodically invoked as the possible cause of TSEs) that may team up with the prion protein to cause disease.

The prion conversion reaction

Accumulation of an abnormal isoform (PrP^{Sc}) of the host encoded PrP^C in the central nervous system is a hallmark of prion diseases. Because no chemical differences were found between PrP^C and PrP^{Sc}, the two species are believed to differ in their conformation. The 3D structure of PrP^C has been determined:[24–27] this has allowed for interesting insights and speculations regarding the mechanism of PrP^{Sc} formation in familial spongiform encephalopathies. However, it has never been possible to determine the high-resolution structure of PrP^{Sc}: all we know is that the β-sheet content of PrP^{Sc} is much higher (43%) than that of PrP^C (3%).[28] Conversion of PrP^C into PrP^{Sc} in prion-infected cells is known to be a later post-translational process, occurring after PrP^C has reached its normal extracellular location. In order to explain the mechanism by which a misfolded form of PrP could induce the refolding of 'native', normal PrP molecules into the abnormal conformation, two distinct models have been postulated: (i) the template assistance or 'refolding' model, and (ii) the nucleation-polymerization or 'seeding' model

(Figure 1). In the first model, the conformational change is kinetically controlled; a high activation energy barrier prevents spontaneous conversion at detectable rates. Interaction with exogenously introduced PrP^{Sc} causes PrP^C to undergo an induced conformational change to yield PrP^{Sc}. This reaction may involve extensive unfolding and refolding of the protein to explain the postulated high energy barrier and could be dependent on an enzyme or chaperone, provisionally designated as protein X.[29] In the second model, PrP^C and PrP^{Sc} are in equilibrium strongly favoring PrP^C. PrP^{Sc} is only stabilized when it adds onto a crystal-like aggregate of PrP^{Sc} acting as a seed in nucleation-dependent polymerization process.[30] Consistent with the latter model, cell-free conversion studies indicate that PrP^{Sc} aggregates are able to convert PrP^C into a protease-resistant PrP isoform.[31−34]

PrP^C and PrP^{Sc}

The cellular prion protein PrP^C contains two N-linked complex-type oligosaccharides at positions 181 and 197. Accordingly, Western blot analysis of PrP reveals three major bands, reflecting PrP that has two, one, or no glycosylation signals occupied. Glycosylation is important as a signal for correct intracellular trafficking of PrP^C and possibly also for selective targeting of PrP^{Sc} to specific brain regions.[35,36]

Upon translocation of PrP into the endoplasmic reticulum an NH$_2$-terminal secretory signal peptide of 22 amino acids is cleaved from PrP, and 23 COOH-terminal residues are processed during addition of the GPI-anchor to the amino acid residue serine 231. Mature mouse PrP contains 209 amino acids. PrP^{Sc}, which accumulates in TSE-affected organisms is a modified form of PrP^C. PrP^C and PrP^{Sc} have an identical amino acid sequence and share the same posttranslational modifications as assessed by currently available methodology, but differ in their secondary and (presumably) tertiary structure. The physiological isoform PrP^C is protease-sensitive (consequently sometimes designated as PrP^{Sen}), while the pathological isoform PrP^{Sc} is partially protease-resistant (thus also called PrP^{res}). The protease-resistant core of PrP^{Sc}, designated PrP$^{27−30}$ according to its molecular weight on SDS−PAGE, provides a specific and reliable molecular marker for the presence of the infectious agent (Figure 2).

Because of its location at the outer surface of cells, anchored by phosphatidylinositol glycolipid, PrP is a candidate for signaling, cell adhesion or perhaps even for some transport functions. PrP is expressed on many cell types, including neurons, glial cells, and lymphocytes[37−39] and appears to be developmentally regulated during mouse embryogenesis.[40] All prion proteins investigated hitherto
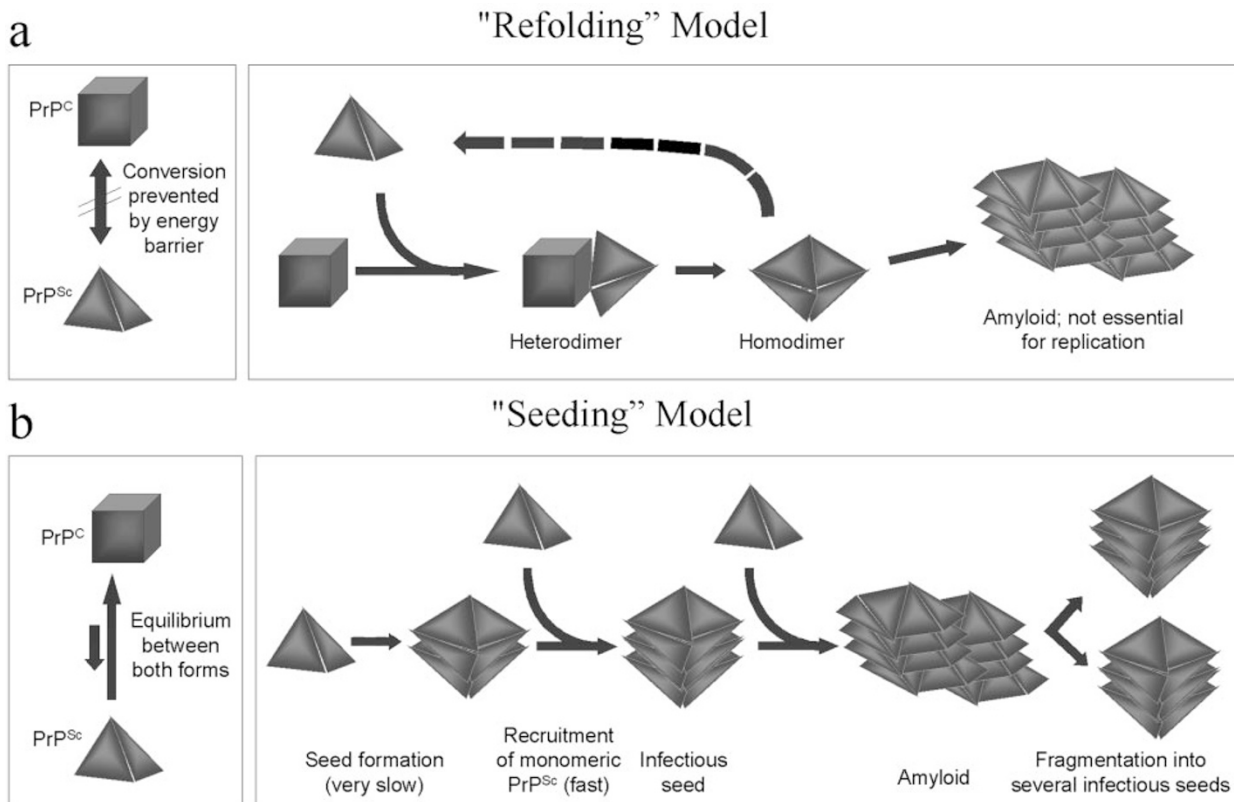
Figure 1 Models for the conformational conversion of PrP^C into PrP^{Sc}. (**a**) The 'refolding' or template assistance model postulates an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^C, which is induced to transform itself into further PrP^{Sc}. A high energy barrier may prevent spontaneous conversion of PrP^C and PrP^{Sc}. (**b**) the 'seeding' or nucleation-polymerization model proposes that PrP^C and PrP^{Sc} are in reversible thermodynamic equilibrium. Only if several monomeric PrP^{Sc} molecules are mounted into a highly ordered seed, further monomeric PrP^{Sc} can be recruited and eventually aggregates to amyloid. Within such a crystal-like seed, PrP^{Sc} becomes stabilized. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit further PrP^{Sc} and thus results in apparent replication of the agent
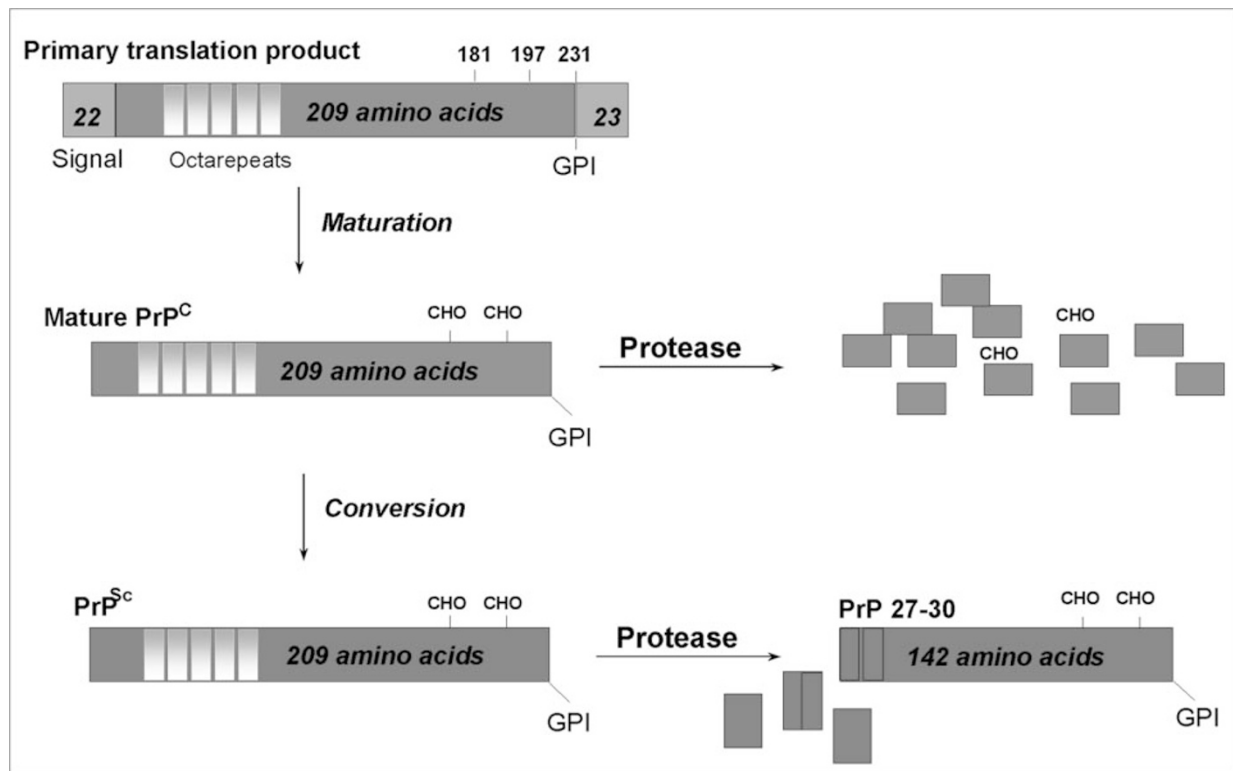
Figure 2 Posttranslational processing of PrP^C. The coding region of the human PrP gene displaying the amino- and carboxy-terminal signal sequences; the five light grey boxes indicate the 'octa repeats', sequences of 8 amino acids. Upon reaching its destination on the cell surface, an amino-terminal secretory signal peptide of 22 amino acids is cleaved from the 254 amino acid PrP^C precursor protein. Twenty-three carboxy-terminal residues are also processed during addition of the GPI-anchor to a serine residue at position 231. Upon completion of these modifications, mature PrP^C contains 209 amino acids. PrP^{Sc} is a modified form of PrP^C. PrP^C and PrP^{Sc} have an identical amino acid sequence and share the same posttranslational modifications, but differ in their secondary and (presumably) tertiary structure. The physiological isoform PrP^{Sc} is protease-sensitive, while the pathological isoform PrP^{Sc} is partially protease-resistant displaying a protease-resistant core of PrP^{Sc}, designated PrP$^{27-30}$

contain four to five 'octa repeats'; repetitive sequences of eight amino acids.[41] Amplification of the number of octa repeats has been found in hereditary prion diseases such as familial Creutzfeldt-Jakob disease (fCJD) and Gerstmann-Sträussler-Scheinker syndrome.[42,43] Mammalian and chicken prion octa repeats bind up to four copper atoms. PrP refolded with copper has been shown to have an antioxidant activity similar to that of superoxide dismutase. It has been suggested from studies with peptides based on the octa repeat region of PrP that the four highly conserved histidines in the repeat region coordinate four copper atoms, suggesting that binding of copper may be essential for the function of PrP^C.[44] Though it remains to be clarified whether the SOD-like activity of the PrP-copper complex is primarily due to an induction of PrP under conditions of oxidative stress involving a binding to copper.

Although PrP is predominantly found in brain tissue, high levels are also present in the heart, skeletal muscle and kidney whereas it is barely detectable in the liver.[45] Several candidate proteins that bind PrP^C have been reported. Among them are a member of the amyloid precursor protein (APP) family, the amyloid precursor-like protein 1 APLP1,[46] the 37 kDa human laminin receptor precursor[47] and an uncharacterized 66-kDa membrane protein.[48] The observation that PrP binds to APLP1 is intriguing in light of

potential analogies of transmissible spongiform encephalopathies to Alzheimer's disease. Evidence that any of these interactions are of physiolgical relevance is, however, still lacking.

Prnp – the gene encoding PrP

The gene encoding the cellular isoform of the prion protein is located on chromosome 20 in humans and on chromosome 2 in mice. It has been cloned by Charles Weissmann and termed Prnp in mouse and PRNP in humans.[4] In all known mammalian and avian Prnp genes, PrP^C is encoded by a single open reading frame. The Prnp genes, of mice, sheep and cattle contain three exons, while those of hamsters and humans span over two exons.[49] PrP expression seems to be controlled by the SP1 transcription factor that binds to G+C rich nonamer-regions upstream of exon 1.[50] Within the genome, Prnp is present as a single copy gene and does not show any homologies to other known cellular genes. Prnp mRNA is constitutively expressed in the adult organism and developmentally regulated during mouse embryogenesis.[40] Mutations in the host-encoded PrP gene were shown to be genetically linked to human prion diseases. In addition, the PrP gene controls many features of prion diseases such as incubation time, species barrier, and strain specificity.

Disruption of the *Prnp* gene demonstrated that an organism that lacks PrP^C is resistant to infection with prions.[7]

Prnp and *Prnd*: double trouble?

Different knockout strategies for disrupting *Prnp* have been employed. For instance, the so-called Zurich I Prnp$^{0/0}$ mice as well as the Edinburgh *Prnp*$^{-/-}$ used similar approaches thus deleting two thirds of the open reading frame (ORF;[6] Manson, 1994 #470]. These mice were clinically healthy, although displaying discrete neurophysiological changes and demyelination of peripheral nerves on aging. However, mice of a third PrP knockout line, Nagasaki Prnp$^{-/-}$ developed ataxia and loss of cerebellar Purkinje cells.[51,52] Since introducing a PrP-encoding cosmid prevented the disease, this phenotype was attributed to PrP.[53] In contrast to the Zurich I and Edinburgh PrP knockout mice, in the Nagasaki line, not only the ORF, but also parts of intron 2 and the non-coding flanking regions were disrupted. Recent work possibly providing an explanation for the Nagasaki-mice-related phenotype: sequencing of a *Prnp*-containing cosmid revealed an ORF encoding a protein, christened Doppel (Dpl), 16 kb downstream of *Prnp*.[54] Doppel − encoded by a gene termed *Prnd* − displays ∼25% identity with the carboxy proximal two thirds of PrP, but lacks a sequence homologous to the amino proximal copper-binding octa repeat region and in particular the highly conserved region 106−126 of PrP, which is essential for its capacity to sustain prion replication. Interestingly, Dpl mRNA is expressed at very low levels in brain of wild-type mice, but − and this could explain the phenotype described above − there are high expression levels in the brain of Nagasaki mice.

Interestingly, *Prnp*$^{0/0}$ mice with an amino proximally truncated transgene encoding PrP devoid of the octa repeats and the conserved 106−125 region suffer from ataxia and degeneration of the cerebellar granule cells shortly after birth.[55] Introduction of a single intact PrP allele rescues this phenotype. Based on these findings it is thought that PrP interacts with a ligand to obtain an essential signal. In the absence of PrP a notional Prp-like molecule with lower binding affinity possibly substitutes for PrP function. According to this hypothesis, in PrP knockout mice, by interacting with the ligand the truncated PrP could displace the PrP-like molecule, though not eliciting the survival signal. If PrP has the highest affinity for the ligand, it would replace its truncated equivalent and restore function. Because Dpl resembles the truncated PrP, it might cause disease by the same mechanism[56,57] (Figure 6b).

Mechanism of cell death

One of the issues that is poorly understood is how and why prions may actually lead to neurodegeneration. The cellular prion protein has been shown to specifically interact with the proto-oncogene product Bcl-2 in the yeast two-hybrid system.[58,59] Bcl-2 is known to suppress cell death in various cell types.[60] Interestingly, in contrast to cells derived from a hippocampal cell line expressing PrP$^{+/+}$ the removal of serum from PrP$^{-/-}$ neurons causes apoptosis that is suppressed by transducing PrP$^{-/-}$ cells with either PrP or Bcl-2.[61] Although

there is no evidence for direct binding of PrP^C and Bcl-2 in vertebrate cells, these experiments suggest a functional interaction between PrP^C and Bcl-2 supporting the idea that a loss of PrP^C function might trigger apoptotic cell death in prion diseases (Figure 6b).

The species barrier in prion diseases

Transmission of prions from one species to another is usually accompanied by a prolongation of the incubation period in the first passage and incomplete penetrance of the disease. Subsequent passage in the same species occurs with high frequency and shortened incubation times.[62] In the case of prion transmission from hamster to mice, this so-called species barrier was overcome by introducing hamster *Prnp* transgenes into recipient wild-type mice.[63] Crucially, the properties of the prions produced were compatible with the prion species used for inoculation: infection with hamster prions led to production of hamster prions, whereas infection with mouse prions gave rise to mouse prions. With respect to the protein-only hypothesis these findings can be interpreted as follows: hamster PrP^C but not murine PrP^C (the latter differing from the former by 10 amino acids) is an appropriate substrate for conversion to hamster PrP^C by hamster prions and vice versa.[64] Intriguingly, susceptibility of mice to prions from other species is increased when the corresponding PrP transgene is introduced into a PrP knockout mouse, suggesting that the resident murine gene inhibits the propagation of the alien prions.[7]

Perplexing prion stains

Distinct isolates or strains of prions were first described in scrapie-diseased goats, where two dissimilar clinical manifestations ('scratching' and 'drowsy') were identified.[65] These strains differ in their incubation times in various inbred mouse lines and by their lesion pattern in the brain. Strikingly, distinct strains of prions can be propagated in an inbred mouse strain that is homozygous with respect to *Prnp*[66] − a perplexing finding with regard to the protein-only hypothesis meaning that an identical polypeptide chain is able to mediate different strain phenotypes. Both the 'refolding' and the 'seeding' model propose that each strain is associated with a distinct conformation of PrP^{Sc} and that each of these can convert PrP^C of its host into a likeness of itself. Indeed, PrP^{Sc} species associated with two hamster-adapted scrapie strains, namely hyper (HY) and drowsy (DY), proved to display characteristic clinical and histopathological properties as well as distinct biochemical patterns with respect to proteinase K digestion[67] − readily explainable by the presence of different conformations of PrP^{Sc}. Analogous findings have been made with other prion strains propagated in mice.[10,68] In addition, PrP^{Sc} of certain strains differs in the ratio of diglycosylated to monoglycosylated form.[13] Based on the fragment size and the relative abundance of individual bands, three distinct patterns (PrP^{Sc} types 1−3) were defined for sporadic and iatrogenic CJD cases. In contrast, all cases of nvCJD displayed a novel pattern, designated as type-4 pattern (Figure 3). Interestingly, brain-extracts from BSE-infected cattle as well as BSE-inoculated macaques exhibited a type-4
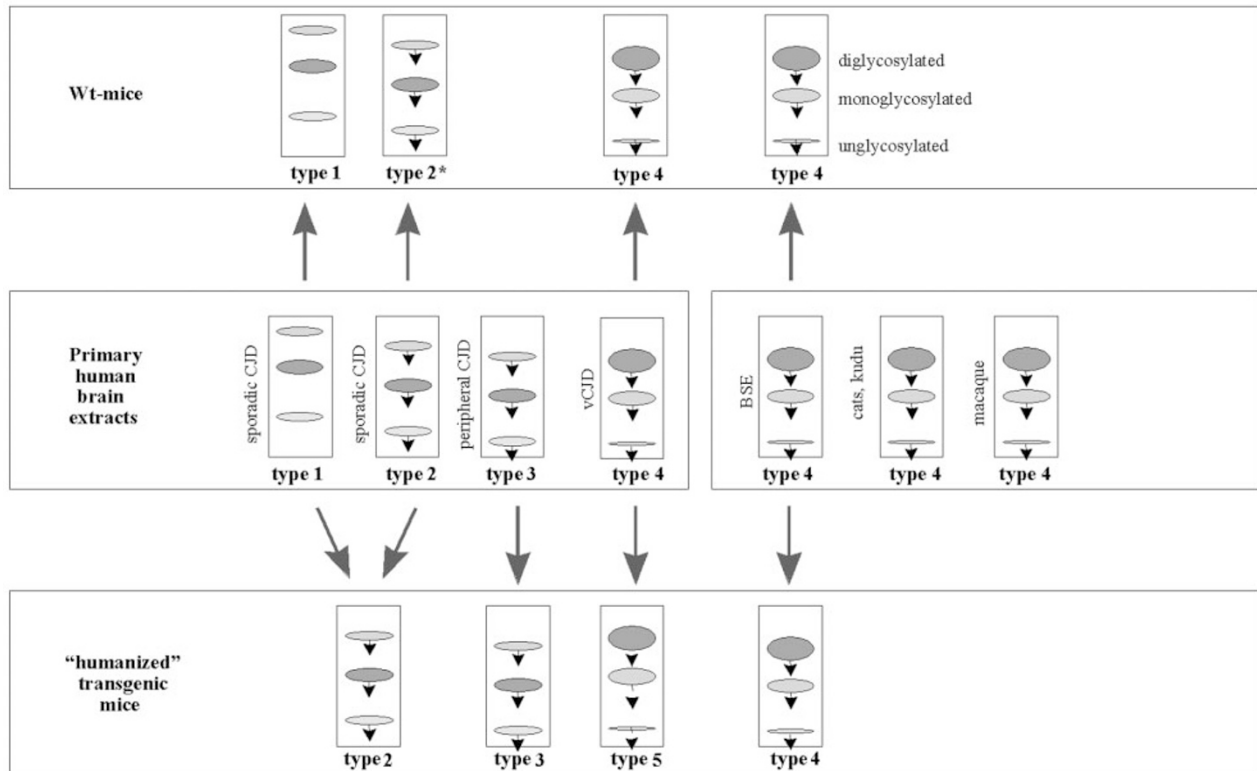
Figure 3 Patterns of PrP glycosylation. Representation of the three glycosylated PrP^{Sc} moieties (non-, mono-, and diglycosylated PrP^{Sc}) in immunoblots of brain extracts after digest with proteinase K. Different inocula result in specific mobilities of the three PrP bands as well as different predominance of certain bands (middle panel). These characteristic patterns can be retained, or changed to other predictable patterns after passage in wild-type (upper panel) or humanized mice (PrP-deficient mice bearing a human PrP transgene, lower panel). Based on the fragment size and the relative abundance of individual bands, three distinct patterns (PrP^{Sc} types 1–3) were defined for sporadic and iatrogenic CJD cases. In contrast, all cases of nvCJD displayed a novel pattern designated as type-4 pattern

pattern. Even more intriguingly, transmission of BSE or nvCJD to mice produced mouse PrP^{Sc} with a type-4 pattern indistinguishable from the original inoculum[10] – findings that impressively support the hypothesis that nvCJD is the human counterpart to BSE. A more powerful tool (eventually serving as diagnostic tool to characterize prion strains) has been provided by the group of Prusiner utilizing the differential affinity of anti-PrP^{Sc}-antibodies, thus differentiating as many as eight different strains.[69] Also, this provides further evidence for the assumption that strain specificity is encrypted within the physical structure of PrP.

Studies of scrapie in inbred mice led to identification of a single autosomal gene, termed *Sinc* (*scrapie incubation*), controlling incubation time of mice infected with mouse prion strains.[70,71] Soon after, a link between *Sinc* and a polymorphism in the mouse *Prnp* gene was discovered and it has been demonstrated that *Sinc* is identical with to *Prnp* by exchanging *Prnp* alleles of inbred mice.[72]

Clinical and histopathological characteristics of prion diseases

Sheep scrapie is the prototype of the growing group of TSEs. The typical symptoms of scrapie-sick sheep include hyper-

excitability, pruritus and myoclonus. The disease is characterized by a rapid progression leading to tetraparesis and ultimately to the death of the affected animal.

The clinical symptoms of BSE are insidious, and consist of behavioural changes (including aggressive behaviour, which is proverbially atypical in cows), and uncoordinated gait. Histologically, the brain exhibits lesions similar to those of TSEs in other species.[73] It has not been excluded that cases of BSE may have been seen in England as early as 1985, but probably not before that year despite earlier anecdotal reports. The number of cases continued to increase, resulting in a major epidemic, and peaking in the summer of 1992.[21]

It was suggested early on that one common exposure of cattle to prions came about through the use of a dietary protein supplement, meat and bone meal (MBM), that was regularly fed to them after weaning. No credible alternative hypothesis has been put forward regarding the origin of the BSE epidemic, and the incidence of new cases has been precipitously declining some 4–5 years after a more-or-less effective ban of MBM was put into place.
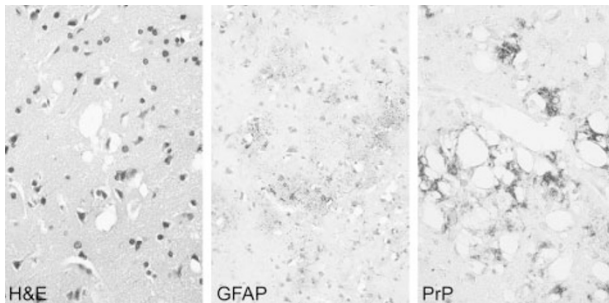
Since the first description of CJD by Jakob and Creutzfeldt,[74] five human diseases have been identified as TSEs (Table 1).[11,12,75] A striking hallmark applying to all

TSEs is that the brain is heavily affected in sharp contrast to the body that remains unharmed. To ultimately diagnose human prion diseases, a histopathological assessment of the central nervous system is essential. The communal lesions are neuronal loss, spongiosis and astrogliosis, accompanied by an accumulation of microglia and, occasionally, the presence of amyloid plaques and various kinds of small deposits immunolabeled with anti-PrP antibodies (Figure 4).[11,76,77] These deposits are variable in their number and topography, hence representing one of a number of phenotypic variations typical of the various forms of human prion disease. Thus, future classifications of these disorders are likely to incorporate genetic and biochemical data in addition to clinical and pathological parameters. CJD occurs sporadically with an incidence of approximately one case per million per year, may be transmitted and has a genetic basis in roughly 10% of all cases. Clinically, patients suffering from CJD show a wide spectrum of diversely associated symptoms. Besides the typical rapidly progressive form including dementia, myoclonus, cerebellar ataxia, visual disturbances and periodic sharp waves seen with electroencephalography (EEG), other forms of the disease exist and may give rise to diagnostic difficulties. Albeit not diagnostic, periodic EEG or 14-3-3 protein detection in spinal fluid are helpful in making the diagnosis, when clinical symptoms are present. Interestingly, there are no general symptoms such as fever or leukocytosis reminiscent of a humoral immune response.[75,78]

The most recently recognized form of CJD in humans, new variant CJD (nvCJD), was first described in 1996 and has been linked to BSE.[79] It represents a distinct clinico-pathological entity that is characterized at onset by psychiatric abnormalities, sensory symptoms and ataxia, eventually leading to dementia along with other features usually observed in sporadic CJD. What distinguishes nvCJD from sporadic cases is that the age of patients is abnormally low (nvCJD: 19–39 years; sporadic CJD: 55–70 years) and the duration of the illness is rather long (nvCJD: 7.5–22 months; sporadic CJD: 2.5–6.5 months). Moreover, new variant CJD displays a distinct pathology within the brain characterized by abundant 'florid plaques', decorated by a daisy-like pattern of vacuolation. Most cases of nvCJD have been observed in the UK.
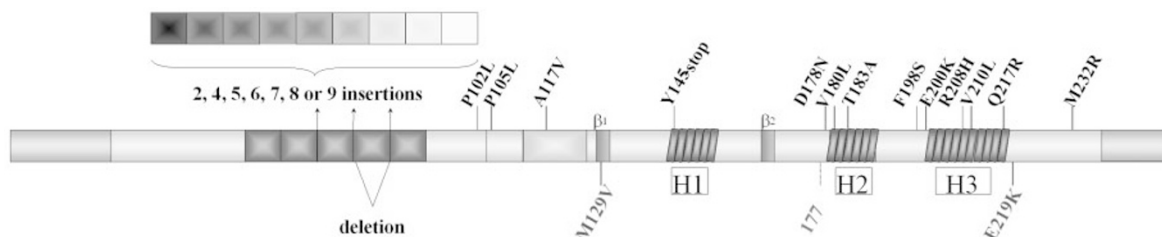
Other genetic diseases in humans are Gerstmann-Sträussler-Scheinker (GSS) syndrome and fatal familial insomnia (FFI). GSS is transmitted by autosomal dominant inheritance.[80,81] It is characterized by missense mutations of the gene encoding PrP (PRNP, Figure 5),[82] associated with specific neuropathological lesions, and by multicentric amyloid plaques that can be visualized by antibodies directed against the prion protein. This restrictive definition justifies retaining the name of Gerstmann-Sträussler-Scheinker syndrome and excludes observations of heredi-tary prion diseases without multicentric amyloid plaques, as well as sporadic forms with multicentric plaques. It has been possible to transmit GSS to chimpanzees and to mice. An additional member of the group of TSEs, FFI, was also transmitted experimentally, in at least three instances to mice.[83] Kuru is a form of spongiform encephalopathy transmitted by ritual cannibalism, which affected the Fore population in the Northern provinces of New Guinea.[84]

Acquired forms of prion diseases in humans (Table 2)

Direct transmissions of brain-derived material from a patient suffering from Creutzfeldt-Jakob disease to other persons

Figure 4 Characteristic neuropathologic features of transmissible spongi-form encephalopathies. Grey matter of the brain of a CJD patient stained with Hematoxylin-Eosin (HE) displaying the characteristic spongiform, vacuole-like morphology (left). Activation and proliferation of reactive, swollen astrocytes is visualized by staining with antibodies against glial fibrillary acidic protein (GFAP, middle). Immunohistochemical staining using anti-PrP antibodies representing prion protein deposits (right)

Figure 5 Schematic drawing of the coding region of the human PRNP gene. Mutations that segregate with inherited prion diseases are shown above, as well as non-pathogenic polymorphisms (M129V, E219K) and silent mutations (177). The N- and C-terminal domains are signal peptides that are cleaved off during maturation of PrP^C (see also Figure 2). Octa repeat regions are represented by grey boxes, and pathogenic octa repeat insertions of 16, 32, 40, 48, 56, 64 and 72 amino acids are shown above. Deletion of one octa repeat stretch does not appear to segregate with a neurodegenerative disorder. There is an apparent clustering of pathogenic mutations associated with a CJD phenotype around the α-helical domains (H1, H2, H3) while amino acid exchanges associated with a GSS phenotype are located further upstream around the conserved region

Table 2 Acquired human prion diseases

Disease	Mechanism of pathogenesis
Iatrogenic CJD	prion-contaminated electrodes human pituitary growth hormone human pituitary gonadotropin dura mater grafts cornea grafts
New variant CJD	infection from BSE prions (hypothesized)

were proven to result in transmission of disease, when electrodes used for cortical recordings from Creutzfeldt-Jakob patients were sterilized using formaldehyde vapors and alcohol, and used in additional patients. Moreover, several thousands of patients, predominantly in Japan, may have been exposed to the CJD agent via preparations of prion-contaminated dura mater, although less than 2% of those exposed have developed disease so far.[85,86] Also, corneal transplantation has most likely resulted in transmission of the disease in the USA two decades ago. The largest problem with iatrogenic transmission of CJD occurred as result of peripheral administration of pituitary hormones of cadaveric origin.[86,87] Nonetheless, iatrogenic transmission is a rare event. This can be explained in two ways: either by the long incubation time of prion diseases – especially in the above described case of extracerebrally administered growth hormone – assuming that further cases may arise in the future; or by some host factors, in addition to the virulence of prions, which may affect the probability that infection takes place, thus either protecting or sensitizing individuals exposed to CJD prions.

The observations that latency after intracerebral contamination (such as cornea and dura mater transplants) is much shorter than latency after peripheral infection (as in the case of extracerebral application of pituitary hormones) is in agreement with experimental data from animal models, and suggest that a rather prolonged period of extracerebral events possibly including replication of the agent and invasion of specific extraneuronal systems may be a precondition to prion neuroinvasion.

Prion diseases without PrP^{Sc}

An abnormal PrP (PrP^{Sc}) is a characteristic feature of most prion diseases. However, in some inherited cases the predominant form of PrP detectable in the brain is not PrP^{Sc} but rather ^{CTM}PrP, a transmembrane form of the prion protein.[88] This finding applies to a subset of patients with Gerstmann-Sträussler-Scheinker syndrome, who carry a distinct mutation in the *Prnp* gene, i.e. a substitution of alanine (A) for valine (V) at position 117 (A117V, Figure 5). Interestingly, this mutation, which leads to the irreversible neurodegeneration characteristic of prion diseases, neither produces conventional PrP^{Sc}, nor transmits it to laboratory rodents. Recently, it was shown that the effectiveness of accumulated PrP^{Sc} in causing neurodegenerative disease may indeed depend upon the predilection of host-encoded PrP to be made into the ^{CTM}PrP form.[89] In addition, the time

course of PrP^{Sc} accumulation seems to modulate the events involved in the generation of ^{CTM}PrP, suggesting a common mechanism in the pathogenesis of both genetic and acquired prion diseases[57] (Figure 6b). If true, this hypothesis may also explain why in many instances of human and experimental TSE extremely little PrP^{Sc} can be detected, even in terminal disease.

Neuroinvasion – how prions pave their way to the brain

Acquired forms of prion diseases are by and large transmitted through oral uptake of the agent or peripheral administration (Table 2) – such as intramuscular injection of growth hormone and, to a lesser extent, pituitary gonadotropins – raising the question of how prions find their way to the CNS.

Neuroectodermal tissue of *Prnp*$^{+/+}$ mice postnatally grafted into the brain of *Prnp*$^{0/0}$ mice develops into differentiated *Prnp*$^{+/+}$ nervous tissue. Intracerebral inoculation of such engrafted mice leads to typical scrapie pathology within the graft but not in the surrounding *Prnp*$^{0/0}$ tissue, while intraperitoneal infection does not, suggesting that prion transport from periphery to CNS requires interposed PrP-bearing tissue.[90,91] Since no pathology can be identified in organs other than the CNS it is of great importance to identify 'reservoirs' within the periphery in which prions (silently) multiply during the incubation phase of the disease.

One such reservoir of PrP^{Sc} or infectivity is doubtlessly the immune system, and a wealth of early studies points to the importance of prion replication in lymphoid organs.[92,93] The nature of the cells supporting prion replication within the lymphoreticular system, however, is still uncertain. Inoculation of various genetically modified immunodeficient mice lacking different components of the immune system with scrapie prions revealed that the lack of B-cells renders mice unsusceptible to experimental scrapie.[94] While defects affecting only T-lymphocytes had no apparent effect, all mutations affecting differentiation and responses of B-lymphocytes prevented development of clinical scrapie. Since absence of B-cells and of antibodies correlates with severe defects in follicular dendritic cells (FDCs), the lack of any of these three components may prevent clinical scrapie. Yet mice expressing immunoglobulins exclusively of the IgM subclass without detectable specificity for PrP^C, developed scrapie after peripheral inoculation: Thus, differentiated B-cells seem crucial for neuroinvasion of scrapie regardless of B-cell receptor specificity. FDCs have been incriminated, because PrP^{Sc} accumulates in FDCs of wild-type and nude mice (which suffer from a selective T-cell defect).[95] Additional intriguing arguments for a crucial role of FDCs are presented by recent findings demonstrating that switching off FDCs (by treating mice with soluble lymphotoxin-β receptor) abolishes splenic prion accumulation and retards neuroinvasion, thus indicating a central function for FDCs in either accumulating or even propagating prions.[96]

Repopulation of immunodeficient mice with fetal liver cells (FLCs) from either PrP-expressing or PrP-deficient mice and

from T-cell deficient mice, but not from B-cell deficient mice, is equally efficient in restoring neuroinvasion after intraperitoneal inoculation of scrapie prions.[94] This suggests that cells whose maturation depends on B-cells or their products (such as FDCs) may enhance neuroinvasion. Alternatively, B-cells may transport prions to the nervous system by a PrP-independent mechanism, since PrP expression in B cells is not required for prion neuroinvasion.[97]
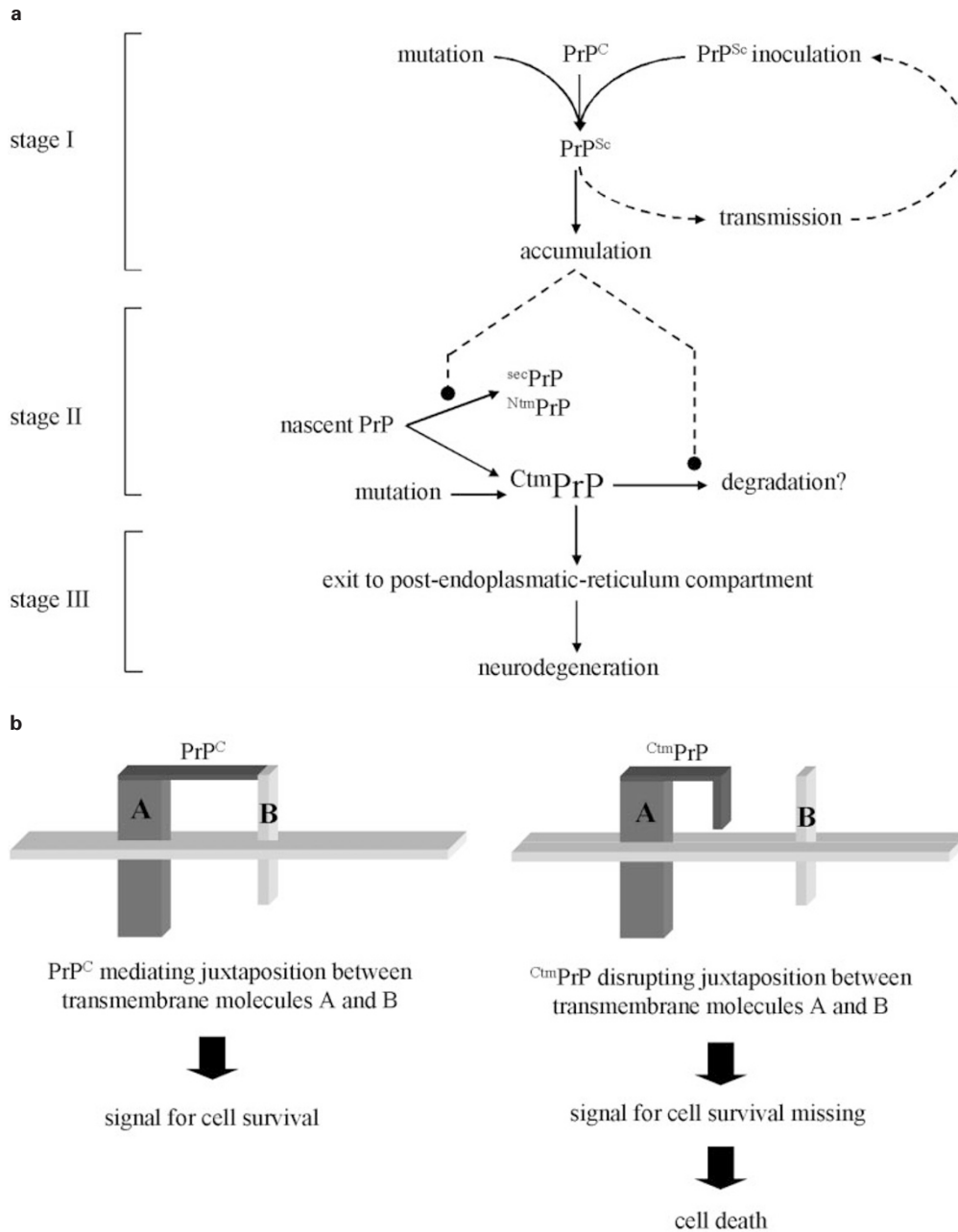
Figure 6 (**a**) Three stage model of prion pathogenesis (adapted from[89]). Stage I represents the formation and accumulation of PrP^{Sc}, initiated by either inoculation or spontaneous conversion of a mutated PrP^C to PrP^{Sc}. Stage II symbolizes the events involved in generating ^{Ctm}PrP, either by an unknown process involving PrP^{Sc} (characterized by dashed lines) or by certain mutations within PrP. Two distinct forms of PrP can be made at the endoplasmatic reticulum: one that is fully translocated (^{sec}PrP) and one that is transmembrane. Digestion with proteases of the latter one results in two fragments: one is COOH-terminal derived and glycosylated, the other is NH$_2$-terminal derived and unglycosylated; thus, the first one is termed C-transmembrane PrP (^{Ctm}PrP) with the COOH-terminus in the ER lumen and the NH$_2$-terminus accessible to proteases in the cytosol, whereas the second one is termed N-transmembrane PrP (^{Ntm}PrP) with an opposite conformation. Stage III hypothesizes on the events involved in ^{Ctm}PrP-mediated neurodegeneration, possibly involving the exit of ^{Ctm}PrP to a post-endoplasmatic-reticulum compartment as a first step that is schematically explained in (**b**). (**b**) Possible role of ^{Ctm}PrP in cell death (adapted from[57]). The full-length PrP possibly acts as a co-receptor on the cell surface, mediating the juxtaposition of two cell-surface transmembrane molecules A and B. This generates a signal for cell survival in the cytosol. Failure of ^{Ctm}PrP to bind B could induce cell death by not facilitating the association of A to B. This mechanism could also explain effects of expression of an amino-terminally truncated PrP(ref.[55]) as well as the Doppel gene product[54]

However, immune cells are unlikely to transport the agent all the way from the lymphoreticular system to the CNS, since prion replication occurs first in the CNS segments to which the sites of peripheral inoculation project. This implies that the agent finally may spread through other compartments, such as the peripheral nervous system (Figure 7).[98,99]

Brain-specific aspects of prion pathogenesis

One of the crucial questions remaining to be answered is why only the brain is affected in prion diseases. Accumulation of PrP^{Sc} is not a brain-specific finding, since one can detect PrP^{Sc} deposits in peripheral organs such as the spleen. While the prime target of damage within the brain seems to be neuronal, profound neuronal loss is not always seen in TSEs. Instead, activation of astrocytes occurs very early and in an extremely consistent fashion (Figure 4).[100] It can be easily reproduced *in vitro*, and leads to significant physiological effects such as impairment of the blood-brain-barrier.[101,102] Additionally, astrocytes belong to the few cell-types identified that are capable of supporting prion replication.[103]

Growing evidence incriminates another cell type in brain damage, not only in TSEs but in diseases ranging from Alzheimer's to multiple sclerosis and even stroke: microglial cells, the brain's intrinsic macrophages.[104] *In vitro* experiments seem to indicate that activation of microglia may be quite pivotal in effecting neuronal damage in TSEs, and that this phenomenon is dependent on expression of PrP^{C}.[105] Moreover, microglial activation and accumulation in affected brain areas precede neuronal cell death and

parallel the temporal and spatial pattern of PrP^{Sc} deposition.[77] The details of this pathway of neuronal cell death, however, still escape our understanding, and the proof that these phenomena occur in the brain during the course of the disease is still missing.

Therapeutic approaches

Because of the unusual properties of TSEs, standard antiviral and antibacterial approaches to these diseases are obviously useless. The situation is further complicated by the fact that there is almost no immune response to infection. Over the past 25 years a variety of different classes of compounds have been tested as inhibitors of TSEs that include antiviral, antibacterial and antifungal drugs, hormones, immunosuppressants and sulfated polyanions. However, none of these substances have been particularly successful: when prion diseases become clinically apparent, damage to the brain is typically extensive, and it is not surprising that pharmacological treatment is ineffective.

Since most cases of TSEs are induced by peripheral infection, and prion titers rise in lymph nodes and spleen before transfer to the CNS, post-exposure prophylaxis, which aims at preventing neuroinvasion, may be feasible. Promising studies involve the use of pentosan polysulphate (PS), a polyanionic sulphated polysaccharide closely related to cell-derived glycosaminoglycans, that recently has been shown to prolong scrapie incubation time and to reduce disease susceptibility in mice.[106] However, PS only showed its protective effect when given both intraperitoneally and at a timepoint close to the i.p. prion infection. Oral administra-
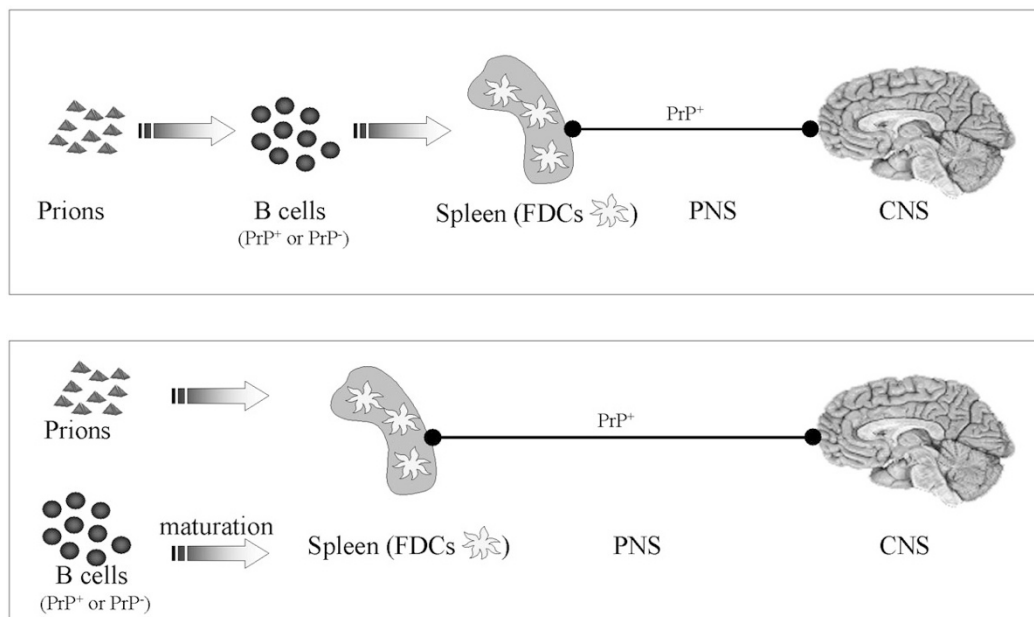
Figure 7 Possible models for prion neuroinvasion. In the upper panel, B lymphocytes, which may be genotypically PrP-positive or PrP-negative, carry physically prions to their sites of replication in spleen. These may be represented, in part or entirely, by follicular dendiritic cells (FDSs). Neuroinvasion proper then occurs preferentially along fibers of the peripheral nervous system (PNS), which may support transfer of prions to the central nervous system (CNS) depending on their own expression of PrP^{C}. The lower panel illustrates a second possibility, i.e. that B cells are not directly involved in neuroinvasion. In this instance, the crucial role of B-cells in neuroinvasive scrapie relies on their function in inducing maturation of FDCs

tion of PS was ineffective, perhaps because of its poor bioavailability upon delivery via the gastrointestinal tract. Since the blood-brain-barrier is practically non-permeable to PS, putative targets of PS must be situated within the periphery. Other potential anti-TSE drugs are certain cyclic tetrapyrroles, i.e. porphyrins and phtalocyanines that have been demonstrated to inhibit the formation of PrP^{Sc} *in vitro*.[107] *In vivo*, these compounds have recently also been shown to delay onset of scrapie in mice when administered over 4 weeks starting at the time of i.p. prion infection.

Of all substances tested, phtalocyanine tetrasulfonate (PcTS), a cyclic tetrapyrrole, displayed the strongest effects in inhibiting PrP^{Sc} formation *in vitro*, and resulted in the most successful delay of disease onset in mice.[108] Again, there was no prolonged incubation time when the drug was given weeks after the prion infection. Another promising approach to delaying scrapie in mice is based on interfering with the conformational transition of PrP^{C} into PrP^{Sc}. Soto and colleagues could demonstrate that a 13-residue β-sheet breaker peptide (iPrP13) partly reversed *in vitro* PrP^{Sc} to a biochemical and structural state similar to that of PrP^{C}.[109]

Treatment of infectious prions with iPRP13 prior to infection delayed onset of clinical symptoms and decreased infectivity by about 90% in mice with experimental scrapie thus possibly representing a novel therapeutic approach to prion diseases. Thus, all putative anti-TSE drugs tested to date are of prophylactic rather than therapeutic usage. Certainly, there is an urgent need for strategies to deal with infected individuals, because millions of people may have been exposed to BSE in the 1980s and possibly in the early 90s.

Yeast prions

For more than 20 years the genetic analysis of two unrelated mutants of the yeast *Saccharomyces cerevisiae* resulted in a substantial dilemma for yeast researchers. The two mutants in question, the [*PSI*$^{+}$] mutant (modifying the efficiency of nonsense suppression) and the [*URE3*] mutant (causing an alteration in nitrogen metabolism) failed to behave according to Mendel's laws. Thus, it was suggested that the unusual genetic properties of both mutants could be explained by a prion-like behavior of the two previously identified yeast proteins: [*PSI*$^{+}$] is associated with Sup35p (also known as eRF3), an essential component of the translation termination machinery, whereas [*URE3*] is associated with Ure2p, a protein that negatively regulates nitrogen metabolism.[110] Lately, further yeast proteins with a prion-like behavior have been discovered; amongst them is Rnqlp fulfilling all diagnostic criteria of yeast prions, although not obviously associated with a specific phenotype.[111]

Strikingly, the prion-forming domain (PrD) of Sup35p very recently has been shown to be both modular and transferable. An artificial prion was generated by fusing a recombinant version of a mammalian receptor to the Sup35p-PrD .[112] The fusion protein altered the biochemical properties of yeast cells in ways that could be inherited by progeny cells. Not only strongly supporting the 'protein only'

hypothesis, the modular and transferable properties of yeast PrD have both fundamental evolutionary and great biotechnological implications if broadly applicable. In a further set of experiments, it was shown that both yeast and a neural tumour cell line cytoplasmatically expressing mouse PrP acquired the characteristics of PrP^{Sc}; that is detergent insolubility and resistance to protease digestion.[113] Studies of infectivity of these transgenetically synthesized prions have not yet been performed, but, in case of a positive outcome, would come close to verifying the protein-only hypothesis. However, unlike the yeast prion-forming domains Sup35p and Ure2p, mammalian PrP is not a cytoplasmic protein. Formation of PrP^{Sc} occurs after it traverses the plasma membrane into a subcellular compartment. Formation of PrP^{Sc} thus occurs in a very different molecular and ionic environment to that in which yeast prions form raising the question of whether a co-player may be involved, namely the so-called protein X. Although the cytoplasmic molecular chaperone Hsp104 is known to be essential for the formation and maintenance of the prion form of Sup35p in yeast, neither Hsp104 nor an analogous chaperone or enzyme – in other words the puzzling protein X – has been proven to be involved in PrP^{Sc} formation. However, whether transgenic mouse PrP^{Sc} expression in yeast is Hsp104-dependent has not been reported.

Overall, yeast is providing a very powerful and experimentally testable paradigm for prions, although caution must be taken in directly extrapolating findings with yeast prions to the mammalian system.

Outlook

Given the wealth of research data on transmissible spongiform encephalopathies, it is tempting to declare that prion diseases constitute the best-understood form of neurodegeneration. Yet it would be deceptive to neglect the fact that most of the mechanisms governing prion replication are not understood: not only the details, but even the very core of the problem is still obscure.

Certainly, understanding of prion pathogenesis has progressed rapidly – but this is a topic that can be often dealt with even in the absence of ultimate certainty about the nature of the infectious agent. However, it is sobering to realize that the prion hypothesis proper is still awaiting irrefutable confirmation. Although each individual piece of evidence (human genetics, transgenic mice, *in vitro* conversion experiments, etc.) strongly supports the proposition that the prion is composed partly or entirely of a PrP-derived molecule and informational nucleic acids are not required for its infectious properties, the available data still does not provide incontrovertible proof of the hypothesis. Equally frustrating are attempts at establishing sensitive diagnostic procedures: the best assays described still do not reach the sensitivity of PCR reactions used for routine detection of viruses. Finally, even if intriguing prionostatic regimens are being developed, we are still very far from an effective and useful proposal for post-exposure prophylaxis or for treatment of prion diseases.

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