Review article

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Prion diseases: a riddle wrapped in a mystery inside an enigma

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Abstract

It is now widely accepted that many structurally diverse proteins can misfold and cause so-called "conformational diseases", including the most common neurodegenerations, Alzheimer's disease and Parkinson's disease. The conversion of largely α-helical or random coil proteins into cross-β-pleated sheet conformations that form first oligomers and then fibrils underlies these disorders. However, this α- to β-structure transition seems to be a generic propensity of all globular proteins, not only those involved in neurodegenerations, not to mention "prion diseases". Metaphorically, all these neurodegenerations are "infectious" in the sense that misfolded β-sheeted conformers are formed in a nucleation process in which preformed metastable oligomer acts as a seed (a nucleus) to convert a normal into an abnormal protein. However, in none but transmissible spongiform encephalopathies (TSEs) has infectivity in a microbiological sense ever been observed, and even in TSEs the formation of misfolded protein is not necessarily accompanied by the generation of infectivity de novo. Furthermore, certain "prion diseases" are not TSEs but just "proteinopathies" caused by accumulation of abnormally misfolded PrP^{d}. The presence of a massive amount of PrP-amyloid and no infectivity casts doubts on whether TSEs are really infectious amyloidoses. The misfolding of PrP may yet prove to be an epiphenomenon secondary to infection with a still unknown infectious agent.

If, on the other hand, the purely proteinaceous character of the replicating unit of TSE infectivity is ultimately found to be correct, the critical issues become 1) the mechanism by which a misfolded PrP template induces normal protein molecules to adopt the same pathologically misfolded conformation, and 2) the intracellular conditions that are responsible for strain differences in these molecules.

Key words: *prion, PrP, virus, transmissible spongiform encephalopathy.*

Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative disorders which include kuru [129], Creutzfeldt-Jakob disease (CJD) [135], Gerstmann-Sträussler-Scheinker (GSS) disease [244], and fatal familial insomnia in man [225,249,250], natural scrapie in sheep, goats [86-89], and mouflons [360], transmissible mink encephalopathy in ranch-reared mink [55], chronic wasting disease of deer, elk and moose in the USA and Canada [155,219,355-357], bovine spongiform encephalopathy or "mad cow disease"

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[347] and its analogues in several exotic species of antelopes [90,119,184,196] and wild felids in zoological gardens [358], and feline spongiform encephalopathy in domestic cats [365].

These disorders are caused by a still not completely understood pathogen variously referred to as a "prion" or a slow, unconventional or atypical virus, or "virino" [3,65,214,215]. Despite wide acceptance of the prion theory, these designations still reflect different views about the real molecular structure of the pathogen and, by the same token, our ignorance of its nature. Those who prefer to view this pathogen as composed "predominantly or exclusively" of a pathologically folded (misfolded) protein (PrP^{res}, PrP^{Sc}; Sc, from scrapie or PrP^{d}; d, from disease; PrP^{TSE}, TSE from transmissible spongiform encephalopathy), use the term "prion" [284,286-288]; hence the term "prion diseases" [65,118,345].

The "virino" hypothesis suggests that the pathogen is a molecular chimera composed of a still-to-be-discovered nucleic acid and a shell protein which is host-encoded (perhaps PrPd) [188-190]. The virus hypothesis simply suggests that the pathogen is a yet-to-be-identified unconventional virus [112,113,232,233,235,236]. The "unified theory" of Weissmann [343,344,346], not unlike the virino theory, suggests that the agent is a molecular chimera of the misfolded protein that confers infectivity and an unidentified oligonucleotide that specifies strain characteristics.

Historical background

Scrapie, a disease of sheep and goats, has been known under several names for some 200 years ("rubbers", "rickets", "goggles", "shakings" "shrewcroft" in England; "scratchie", "cuddie trot" in Scotland; "der Trab", "der Traberkrankheit" or "die Zitterkrankheit" in Germany; "la maladie convulsive", "la maladie follie", "le tremblante", "la prurigo lombaire" in France; and "trzęsawka" in Poland). One of the earliest scientific reports on scrapie was published in the "Agricultural Improvement Society at Bath" (later changed to "Bath and West Society") [10] and, as a paragraph, in the "General View of the Agriculture of Wiltshire" published by Thomas Davies in 1811 [255]. In 1848, Roche-Lubin [293] claimed that scrapie is caused either by sexual excesses of rams or, alternatively, by thunderstorms. M'Gowan [255] himself suggested *sarcosporidium* as the causative agent.

The first interpretation of scrapie ("tremblante") as a virus ("a filterable agent") disease is credited to the French veterinarian Besnoit in 1899 [22], and the transmissible nature of TSEs was proven in 1936 by the seminal experiments of Cuille and Chelle [84-89]. The contention that scrapie is an infectious disease caused by a filterable agent was accepted with scepticism until 1938, when W.S. Gordon, then a deputy director of the Moredun Institute in Edinburgh, Scotland repeated the experiments of Cuille and Chelle using 697 animals, of which some 200 developed scrapie [152,278]. Ironically, the infectious nature of the scrapie agent had already been inadvertently confirmed in 1935, when some 7% of 18 000 sheep vaccinated against louping ill developed scrapie [151-153]. The vaccine had been produced from sheep brains and also showed that scrapie infectivity could survive 0.35% formalin for more than 3 months. A similar error was repeated recently [338,367]. Interrupted by World War II, scrapie research was continued by D.R. Wilson [359], who was reluctant to publish data on such an unorthodox pathogen, but the scrapie community was well aware of the unusual properties of the scrapie agent, in particular its high resistance to formalin and heat.

Scrapie was transmitted from goats to mice by Chandler [69-72] and from sheep to mice by Morris and Gajdusek [262], enabling more convenient laboratory research, and resulting in new hypotheses about the nature of the causative agent every year or two. It was proposed to be a self-replicating membrane [8,134, 174-176] or a subvirus (not well envisaged) linked to a membrane with a "linkage substance" [1,2], a viroid [109-111,226,227,238,241-243], a spiroplasma [15-19], or a retrovirus-like element [5-7,230,320,332]. Suffice to say that none of those hypotheses could be substantiated despite exhaustive use of all methods of both classical and molecular virology, yet the virus hypothesis remains plausible [232].

The first TSE in humans was kuru, discovered by Gajdusek and Zigas [128,213,214,218]. The elucidation of kuru opened a new field in human medicine and initiated more than 50 years of research which has contributed enormously to our understanding of neurodegenerative disorders of the central nervous system, including Alzheimer's disease [126,127,204,218]. Kuru was transmitted to chimpanzees in 1965 [129], and was quickly followed by transmission of Creutzfeldt-Jakob disease [135] and GSS [244]. The most recent transmission of a human TSE was fatal familial insomnia [81,333].

In 1987, Gerald A.H. Wells and his colleagues described a cow with a novel form of TSE [347] (parenthetically, the first case of BSE had actually been observed in a nyala by Jeffrey et al. [184]). The BSE

epidemic reached a climax in 1992 with more than 37 000 cases, and subsequently steadily declined to 612 in 2003 and x in 2005 [34,35,78]. However, in 1996 Will et al. [353] reported a new variant of CJD (vCJD), suggesting on the basis of epidemiological evidence that it was due to a BSE infection, which was subsequently substantiated by laboratory studies [52,82,164,312]. Although the number of vCJD cases is decreasing (Table I) [165], and the shape of the outbreak appears to be duplicating that of BSE at an interval of 7-8 years, their occurrence has accelerated TSE research and transformed it from a rather obscure field into a major scientific endeavour.

Nomenclature

The nomenclature of PrP species is confusing. PrP^{c} is a normal cellular isoform. PrP^{Sc} (PrP^{res}) is a pathological misfolded protein. PrP^{Sc} is operationally defined as resistant to proteinase K (PK) and insoluble in denaturing detergent; however, some pathological isoforms of PrP have recently been found not to be PK-resistant [123]. Thus, we prefer to use the neutral term PrP^{d}, which denotes that misfolded species of PrP which is *disease-associated*, PK-resistant or not, or PrP^{TSE}. PrP 27 – 30 is a proteolytic cleavage product of PrP^{d} [31,245].

Table I. Number of definite and probable cases in the UK, up to 2nd May 2008 (Source: National CJD Surveillance Unit. www.cjd.ed.ac.uk; modified)

Year	sCJD	iCJD	fCJD	GSS	vCJD	Total deaths
1990	28	5	0	0	–	**33**
1991	32	1	3	0	–	**36**
1992	45	2	5	1	–	**53**
1993	37	4	3	2	–	**46**
1994	53	1	4	3	–	**61**
1995	35	4	2	3	3	**47**
1996	40	4	2	4	10	**60**
1997	60	6	4	1	10	**81**
1998	63	3	3	2	18	**89**
1999	62	6	2	0	15	**85**
2000	50	1	2	1	28	**82**
2001	58	4	4	2	20	**88**
2002	72	0	4	1	17	**94**
2003	79	5	4	2	18	**108**
2004	50	2	4	2	9	**67**
2005	66	4	8	5	5	**88**
2006	66	1	6	3	5	**81**
2007	59	2	4	1	5	**71**
2008*	23	2	0	2	0	**27**
Total	**978**	**57**	**64**	**35**	**163**	**1297**

** As at 2nd May 2008; number of definite/probable vCJD cases still alive: – 3; total number of definite or probable vCJD cases (dead and alive): – 166*

PrP, its gene, the "prion hypothesis"

PrP^c is a highly conserved sialoglycoprotein encoded by a cellular gene mapped to chromosome 20 in man and chromosome 2 in mouse [14,37,75,138,157,266, 332,324]. The gene is ubiquitous [136,201,282,339]; it has been cloned in numerous mammalian species, included marsupials, and there are analogues of this gene in birds [124,158,307,308,361] reptiles [319], amphibians [328], and fish [267]; those in Drosophila and nematodes appear to be cloning artefacts [348]. Recently, the *PrP* gene was cloned in several exotic species of mammals – Pekingese dog [363], Amur tiger [362], and African lion [364]. A mammalian gene encoding for PrP demonstrates several interesting features [339]. There are two variants of a signal peptide; the longer one is present in most mammalian orders including marsupials and starts with consensus sequence MVKSH; the shorter one is observed in primates, flying lemur, tree shrew, rabbit and rodents and starts with sequence MAN. The number of repeats varies from 2 (squirrels) to 7 (leaf-nose bat and gynmure). The latter finding is particularly interesting because in humans the expanded number of repeats is linked to familial TSEs (see later). The eutherian repeats are the highly conserved consensus sequence P(Q/H)GGG(g/-)WGQ; the first repeat always has Q. The sequence 143-163 contains a highly conserved hydrophobic transmembrane region. The globular structured region is highly conserved and most substitutions are conservative replacements. In particular, Cys^{216} and Cys^{252} as well as Asn-X-Thr necessary for glycosylation are highly conserved [339].

There are several polymorphic sites within *PRNP* [247]. The best known is Met to Val polymorphism at position 129 in humans [150,269,270]. Of note, the human polymorphic site is not polymorphic in chimpanzees [321]. In Northern European populations, the predominant allele is 129^{Met}, with a frequency of 0.65 [247]; in Iceland 0.46 [132]; in Greece 0.5 [300]; in Poland 0.45 [36]. $129^{Met\ Met}$ is extremely rare in Japan, while it is predominant in the eastern Highlands in Papua New Guinea, with a frequency of 0.55. It is also high in several Northern Indian populations. A 219 codon polymorphism (E219K) was found in Japan [315], while several polymorphisms (G142S; N171S) were found frequently in Africa, but their significance is unknown [247].

Polymorphism at codon 129 influences the susceptibility to human TSEs as well as modifies their phenotypic appearances. The homozygous status of codon 129 ($129^{Met\ Met}$ or $129^{Val\ Val}$) is overrepresented in both sporadic [272] and iatrogenic CJD [80]. Variant CJD is the most distinctive example of this rule – except for a case of vCJD following blood transfusion [280] all other vCJD cases are homozygous $129^{Met\ Met}$ [279]. In contrast, 2 of 3 PrP^d-positive appendix samples (of 11 109 appendices) were $129^{Val\ Val}$ [181]. The latter data strongly suggest that individuals of $129^{Val\ Val}$ and $129^{Met\ Val}$ are also susceptible to vCJD. The situation is thus similar to kuru [68], where individuals of $129^{Val\ Val}$ and $129^{Met\ Val}$ genotype were also susceptible, but those of $129^{Met\ Met}$ genotype were overrepresented in the younger age group, while those of $129^{Met\ Val}$ were overrepresented in the much older age group.

The cause of differences in susceptibility between different genotypes is unclear, but Tahiri-Alaoui et al. [330] demonstrated that rPrP-129^{Met} has a higher propensity to form oligomers enriched in β-sheets, while PrP-129^{Val} shows a higher tendency to fold into α-helices. In contrast, following folding into oligomers, the resistance to proteinase K was the same for both allelomorphs. How the latter phenomenon may be translated into differences in susceptibility, not unexpectedly, is unknown.

The most intriguing role of the homozygocity at codon 129 is its overrepresentation in certain dementive conditions such as Alzheimer's disease [139] or individuals with temporal lobe epilepsy [203]. These findings might suggest that the status of codon 129 may influence some poorly defined aspect of the dementive process that is not entirely specific for TSEs, but might be associated with a process of protein misfolding in general.

PrP is plausibly one of the most extensively studied proteins [64,65]. Human PrP^c contains 253 amino acids encoded by an intronless ORF [14]. Three forms of PrP^c exist – one completely translocated and two rather infrequent, transmembrane variants ^{Ctm}PrP and ^{Ntm}PrP [160,161] – and the sequence encoding residues 151-165 that form the transmembrane region is highly conserved [339]. Furthermore, PrP^c undergoes endoproteolytic cleavage to yield 17 kDa N-terminally truncated form C1, while PrP^{Sc} yields a slightly larger peptide designated C2 [74].

PrP 27-30 was first discovered as a protein co-purifying with infectivity in extracts derived from brains infected with the 263K strain of scrapie agent [31,114, 245], which led to the conclusion that PrP 27-30 is part of infectivity.

The "prion" hypothesis, which is deeply rooted in this association between PrP and infectivity, was

formulated by Stanley B. Prusiner in 1982 [283,285]. The hypothesis postulated that the scrapie agent was a proteinaceous infectious particle, because infectivity was dependent on protein but resistant to methods known to inactivate nucleic acids. A similar proposal had been presented more than a decade earlier by Gibbons and Hunter [134], Griffith [154], and Levine [211], who all developed the earlier suggestion of Tikvah Alper and her co-workers [9], based on irradiation studies, that the scrapie agent was devoid of a disease-specific nucleic acid. While the theoretical approach of Alper et al. was strongly criticised by Rohwer [295,296], Cho and other investigators had found previously that scrapie infectivity was sensitive to proteolytic digestion [77,257].

Like many amyloid proteins, PrP 27-30 is a proteolytic cleavage product of a larger precursor protein, PrP 33-35^{d} [222, 352]. However, PrP 33-35^{d} is not the *primary* product of the cellular gene. It has an amino acid sequence, glycosylinositol phospholipid anchor and posttranslational modifications (such as glycosylation and the attachment of GPI, glycophospholipid inositol anchor) identical to those of PrP 33-35^{c} but strikingly different physicochemical features [268,325,327]; in particular, PrP^{c} is completely degraded by limited proteolysis, but PrP^{d} is only partially degraded, yielding a core protein (PrP 27-30) which may be visualised by electron microscopy as scrapie-associated fibrils (SAF) [252,253], or prion rods [288]. To become PrP^{d}, PrP^{c} must first be transported to the cell surface and then through the endosomal-lysosomal pathway [32,246].

PrP has several interesting features. As already mentioned, it is a glycoprotein with two Asn-glycosylation sites (Asn^{181} and Asn^{197} for HuPrP or Asn^{180} and Asn^{196} for MoPrP) [206,268]; thus, PrP may exist as unglycosylated, monoglycosylated and diglycosylated isoforms of different electrophoretic mobilities and glycoforms [82]. The unglycosylated glycoform comprises 5%, monoglycosylated 25%, whereas the diglycosylated glycoform is dominant, comprising 70% of all glycoforms [268]. However, using 2D-PAGE more than 30 glycoforms were discriminated [326]. There are subtle differences in the relative proportion of different glycans (52 bi-, tri- and tetra-antennary N-linked oligosaccharides) between PrP^{c} and PrP^{Sc} [297,298] that may result from a decrease in the activity of N-acetylglucosaminyl transferase III (GnTIII) toward PrP^{c} [298]. The latter finding may suggest an alteration of the glycosylation pathways in TSEs. Of note, changes in several enzymes involved in carbohydrate metabolism were noticed some quarter of a century ago by Millson and Bountiff [256], Kimberlin and Marsh [192], and Marsh and Kimberlin [239].

The various combinations of glycosylation and codon 129 genotype (see later) correlate to a certain degree with the phenotypic expression of TSE. In particular, a distinctive glycosylation pattern is uniquely present in both BSE and vCJD [82,164,187]. Although glycosylation patterns "breed true", i.e. they are retained in passage [82], interconversions may occur in the presence of metal ions [82,341], and more than one pattern may occur in different regions of the same brain, or in the brain and peripheral organs of the same patient [20]. The role of glycosylation in TSE pathogenesis may be more complex, however. In familial TSE, elimination of the first glycosylation site by a mutation was observed [265]. In contrast, elimination of PrP^{c} glycosylation either by tunicamycin treatment or by artificial mutation of Asn-N-linked glycosylation sites does not prevent PrP^{Sc} formation [199,210,331]. Of note, elimination of PrP^{c} glycosylation results in a peptide with some properties of PrP^{Sc} [199,210] and an unglycosylated peptide (PrP 175-195) rapidly acquires β-sheet conformation and forms amyloid fibrils [33]. As Cancellotti et al. [56] stressed, "the lack of sugar may facilitate TSE onset by inducing PrP to misfold". In contrast, in transgenic mice overexpressing mono-glycosylated (G1: thr → Asp^{180} or G2 Thr → Asp^{196}) or un-glycosylated (both G1 and G2) PrP^{s}, none of PrP^{c} become PK-resistant or insoluble in detergents [56]. In other words, none of PrP^{c} under such experimental conditions acquired PrP^{Sc}-like properties.

There are further glycosylation differences in GPI [325] to yield 6 different isoforms of GPI. Moreover, elimination of the C-terminal sequence encoding the GPI anchor sequence results in a synthesis of unglycosylated protein [294,342]. Collectively, glycosylation alterations may be involved in TSE pathogenesis, but the pathogenesis of that involvement is totally unclear.

Strains of the agent – still the strongest argument against the protein-only hypothesis, but is it really?

From the early work of Alan G. Dickinson and his numerous collaborators [100,101,106-108,121] it was known that the incubation period of scrapie was tightly linked to the gene designated *Sinc* (in mice; from scrapie incubation) and *SIP* (in sheep; from shorter incubation period); Parry [277] even proposed that

scrapie was a primarily hereditary disorder that was only secondarily transmissible (lively discussions between Dickinson and Parry dominated the field in the 1960s) [99]. The discovery of *Sinc* was instrumental in supporting the notion that the scrapie agent has an independent genome [48]. It was subsequently shown that *Sinc* (*SIP*) is identical to the gene encoding PrP^{c} [51], and that $Sinc^{s7}$ is congruent with $Prn\text{-}p^{a}$, while $Sinc^{p7}$ is congruent with $Prn\text{-}p^{b}$ [261]. Furthermore, $Prn\text{-}p^{a}$ ($Sinc^{p7}$) and $Prn\text{-}p^{b}$ ($Sinc^{p7}$) differ by two amino acids in position 109 and 189 [350]. The existence of biologically different strains of scrapie agent is still the strongest argument against the protein-only nature of the scrapie agent; however, recent advances in understanding of the structural biology of PrP may have challenged the latter notion.

Different strains of the scrapie agent can be identified in terms of their stable biological characteristics – those most widely used being the length of the incubation period and the "lesion profile", i.e. semi-quantitative estimation of spongiform change (perhaps resulting from autophagy – 212, 220, 316, 318) in terms of brain topography [49,51,107]. The same strain can be isolated from different hosts and the same host can be infected with different strains. Furthermore, the characteristics of a given strain may sometimes undergo changes to yield a new strain with novel characteristics that are stable in subsequent passages. Such changes would be consistent with the effects of mutations in an (as yet unidentified) disease-specific nucleic acid.

Approximately 20 strains of scrapie agent have been isolated from sheep and goats affected with clinical scrapie [61,94]. Some isolates from sheep yield a mixture of strains. The best known example is the "scrapie sheep brain pool" (SSBP/1) from which 22A, 22C and 22L strains were isolated [60,94]. Some sources of sheep scrapie are not transmissible to mice, for example the CH 1641 isolate [120]; transmissible strains can be divided into two groups on the basis of their properties in two homozygous *Sinc* (*Prn-p*) genotypes of mice. The ME7 group of agents exhibits a short incubation period when passaged through $Sinc^{s7}$ ($Prn\text{-}p^{a}$) mice (s for short; for example C57Bl mice) and a long incubation period when passaged through $Sinc^{p7}$ ($Prn\text{-}p^{b}$) mice (p for prolonged; for example VM mice). The 22A group exhibits exactly the opposite characteristics: short incubation period in $Sinc^{p7}$ mice and long incubation period in $Sinc^{s7}$ mice [96,102-104]. It has been conclusively demonstrated that the *Sinc* gene is congruent with the *Prn-p* gene; in other words PrP is the product of *Sinc* [61,177,191,261]. All recent scrapie isolates differ from the BSE strain, but the spectrum of scrapie strains may have changed over the last 20 years [49].

Passage through a species different from that used for the primary isolation is a useful method to separate mixtures of strains and to isolate (select) new mutant strains [79,193,195]. One of the best known examples of the isolation of a mutant strain with totally different characteristics from the original isolate is the isolation of the 263K (the same as 237sc) strain of the scrapie agent [193-195]. Preservation of strain properties following passage through hosts of different *Prn-p* sequences strongly argues against the protein-only nature of the scrapie agent.

Two sets of experiments may be cited in support of this viewpoint. First, strains of the scrapie agent may undergo phenotypic changes in incubation period, lesion profile, and the presence and amount of PrP^{Sc}-amyloid deposition changes, which are compatible with mutations of "conventional" pathogens [48]. Three classes of strain stability have been established [48]. Class I stability strains (ME7, 22C) possess stable characteristics irrespective of the *Sinc* (s7 or p7) ($Prn\text{-}p^{a}$ or ^{b}) genotype of mice in which they are passaged. Class II strains (22A, 22F) possess stable characteristics if passaged through mice of the *Sinc* genotype in which they were isolated but change these characteristics gradually over several passages through mice of a different *Sinc* mouse genotype. Class III strains (31A, 51C, 87A, 125A, 138A, 153A) exhibit sudden discontinuous changes of characteristics irrespective of the genotype of mice in which they are passaged. All six class III strains are characterised by similar incubation periods, the production of large numbers of amyloid plaques [50], and a high frequency of asymmetrical cerebral vacuolation. It is thus conceivable that all six class III isolates represent the same strain of scrapie agent [48].

"Class III breakdown" was defined as a "sudden shortening of incubation period, in the course of a single mouse passage, accompanied by a marked change in neuropathology" [48]. This usually occurred at some point between the primary and the 7^{th} passage and yielded an isolate designated 7D. The 7D strain was characterised by a shorter incubation period, a more "generalised" lesion profile, and an approximately 10-fold lower frequency of amyloid plaques. All these characteristics are reminiscent of ME7 and it is highly probably that 7D is actually the same as the ME7 strain

of scrapie agent. The ensemble of these data favours the existence of a host-independent scrapie genome.

It must be emphasised that although the emergence of the new strain was independent of the host, its selection was influenced or even governed by the host genotype. Thus, it is misleading to describe two given strains as having "long" and "short" incubation periods because these characteristics are relative and they will depend on the *Sinc* (*Prn-p*) genotype of the mice [343,344,350]. Often the relative incubation periods of two given strains can be reversed on changing the mouse strain [60].

A relevant observation was made following a 1985 outbreak of transmissible mink encephalopathy (TME) in Stetsonville, WI, USA [240]. Two strains of TME had been isolated: one from a hyperactive mink (HY strain), and one from a drowsy mink (DY strain). Both the neuropathological picture and banding pattern of PrP^{d} of DY and HY strains differ [23,24]. Using an *in vitro* PrP^{c} to PrP^{d} conversion reaction [64, 198] it was shown that the conversion is "strain-specific", i.e. HY PrP^{d} only converted HY PrP^{c}, and DY PrP^{d} only converted DY PrP^{c} [25]. This experiment suggested that a certain strain specificity is encrypted within the conformation of PrP itself, which, in turn, determines the site of proteinase cleavage and strain-specific size of PrP fragments using Western immunoblot.

The banding pattern resulting from the conformation of PrP that determines the cleavage site for PK is also regarded as strain-specific, as was subsequently shown by experimental transmission of FFI and CJD to transgenic mice harbouring the chimeric mouse/human transgene [334,335]. Following deglycosylation, the molecular weight of PrP^{FFI} is 19 kDa while that of PrP^{CJD} is 21 kDa [260,275], and the size of PrP^{d} (either 19 or 21 kDa) was retained during passage in Tg mice [334]. These observations were extended by Collinge et al. [82], who showed that the glycosylation pattern of PrP^{d} also "breeds true" and indeed the preservation of this glycotype under passage in Tg mice expressing human PrP on a null background was accepted as "compelling evidence" that BSE and vCJD are caused by one and the same strain [164,312]. As summarized by Caughey [64], these experiments suggest that "different PrP^{d} strains" (i.e. isophorms) can impose their different conformations on a single species of unglycosylated PrP^{c}'. Caughey [64] further suggested that PrP^{Sc} may operate on different pools of PrP^{c}. The latter scenario may reflect different cell (neuronal) populations in which these PrP^{c} molecules reside [20] or additional yet to be discovered ligands. A recent report that nucleic acids promote PrP^{c} misfolding into PrP^{Sc} is extremely interesting in this respect [92].

The most notorious example of a strain of TSE that can be isolated from a different host is the strain which caused vCJD and exhibits the same operational characteristics ("BSE signature") as the causative strain of BSE [52,164,312]. The BSE/vCJD strain is characterised by a distinctive glycosylation pattern [82] that is also seen in the brains of sheep infected with the CH1641 strain of scrapie [166]; however, the vCJD/BSE strain is readily transmissible to mice whereas the CH1641 strain is not [120]. The existence of the CH1641 strain of scrapie underlines the fact that glycosylation patterns may not tell the whole story about strain characteristics. Of note, this "BSE signature" was recently identified in a goat [116].

In scrapie-affected sheep, strains defined by classical methods [102] also have unique glycosylation patterns [323] that differ from BSE [337]. Having said this, recently a new strain of BSE was discovered (BASE) that again differs from classical BSE and, in glycosylation pattern, is more similar to sporadic than variant CJD [26,62]. However, the relation between strains and glycosylation pattern may be even more complex as the highest molecular-weight isoform was found to be full-length diglycosylated "backbone" PrP^{Sc} while lower molecular weight isoforms are either N-terminally truncated PrP^{Sc} or mono- and unglycosylated full-length PrP^{Sc} [273,274]. Furthermore, when PrP^{d} from either sCJD (MM2) or sporadic familial insomnia (also MM2) were compared by 2D immunoblotting, 14 spots were exclusively present in MM2 sCJD samples and all these differences vanished following deglycosylation. Thus, differences between sCJD and FSI (both MM2) are caused by different patterns of glycosylation.

When scrapie-infected mouse brains (ME&, 139A and 22L strains) were probed with Mab 8BG, that recognized full length PrP, 3 bands were seen (34 – 37 kDa, 30 – 31 kDa and 29 kDa) which, following PNGase F treatment, collapsed to a single band of 29 kDa irrespective of the strain investigated [274]. The lower bands are full length underglycosylated PrP species. In addition, two smaller N-terminally truncated PrP species are present in scrapie-infected mouse brains irrespective of the species, but the intensity of those bands varies. Furthermore, the susceptibility to proteolytic cleavage of PrP^{d} is also the same. Collectively, these data suggest that the pattern of PrP^{d} species is not a "fingerprint" of a given species.

The "conformation stability" following denaturation of PrP^d combined with limited proteolysis by PK became a substitute for the tedious measurement of incubation period [209]. Using this methodology Legname et al. [209] found a linear correlation between the incubation period and the $[GdnHcl]_{1/2}$ values that reflect the conformational stability for both "synthetic" and natural "strains". Furthermore, mice with identical incubation times exhibited identical $[GdnHcl]_{1/2}$ values to mice of an extremely long incubation period that also exhibited extremely stable PrP^d.

A second, independent, set of experiments focused on the phenomenon of agent "competition": different strains of the scrapie agent can exhibit competition when inoculated at different times, either intracerebrally (i.c.) [104] or peripherally [103]. For example, when VM mice (*Sinc*^{p7}) were inoculated i.c. with the 22C (slow) strain a week before a second inoculation of the 22A (fast) strain, the mice were killed by the faster 22A strain, as shown by the short incubation period and the characteristic "lesion profile". In contrast, when the time lapse before the second inoculation was prolonged to 9 weeks, the incubation period of 22A increased by 30 days, because of competition with the initially-inoculated slow strain. In another experiment, R III mice (*Sinc*^{s7}), inoculated intraperitoneally with 22A (which now became the slow strain) followed by a second inoculation with 22C (now the fast) strain 100 to 300 days later, did not develop disease caused by the 22C strain. The blocking effect of 22A was so complete that the 22C strain did not produce disease in mice that died after the expected incubation period of 22A. Kimberlin and Walker [194] also studied the blocking phenomenon and showed that the blocking agent must be capable of replication (i.e. infectious).

The results were interpreted as showing two different strains competing for a limited number of multimeric "replication sites" – subunits which are encoded by *Sinc* [96,102-104]. In contrast, when mice were inoculated with the slow (360 days) SY strain of CJD and then 80 days later by the fast (130 days) Fukuoka-1 (FU) strain of GSS, the FU strain was totally blocked and inoculated mice showed no signs of FU disease 250 days post-inoculation but eventually succumbed to the SY strain [234]. On a few occasions, mice showed FU rather than SY pathology suggestive of incomplete suppression of the FU strain and independent replication of either the FU or SY strains against a background of the same PrP sequence [231].

The existence of strains may be explained by a strain-specific oligonucleotide or a ubiquitous virus [232,236]; however, neither has ever been found despite repeated attempts to detect disease-specific nucleic acids [7,186, 254,306]. It must be stressed, however, that even in the most highly purified fractions containing PrP^d, nucleic acids as long as 5000 bp can readily be found [231], which is more than enough to contain a genome of a small albeit otherwise conventional virus [232].

All these data have been interpreted according to the protein-only hypothesis: differences in banding of PrP fragments as seen on Western blot reflect the diversity of PrP^d conformation, so that when PrP^d is formed from misfolded PrP^c in a process of seeding-nucleation in which PrP^d acts as a seed, the existence of strain-specific PrP^d species that "breed true" suggests that strain specificity is encrypted within the conformation of PrP^d. However, there are two caveats to this hypothesis.

First, only a few PrP^d conformers have been identified in association with the approximately 20 strains of scrapie [94], and only two major PrP^d size species (21 kDa type 1 and 19 kDa type 2) have been isolated from human CJD. In GSS, smaller fragments, approximately 8 kDa, were isolated, and recently an sCJD case characterized by the presence of ~6 kDA PrP fragment was reported [202]. Thus, if the existence of strains is reflected merely by the size of PrP^d peptides, all of the diverse phenotypes of CJD and FFI phenotypes are caused by just two strains, which seems highly unlikely. Even the more complicated genotype schema of Collinge et al. [79, 82] offers just a few conformational "strains".

Second, one or the other PrP^d peptide (19 or 21 kDa) may be isolated from different parts of the brain or from brain and lymphatic tissues [276,309], which would mean that the same individual is coincidentally infected with both strains of the agent. Even in vCJD, which has until recently yielded a single glycosylation pattern (i.e. caused by a single strain of CJD according to the "protein-only hypothesis"), two glycosylation patterns have recently been observed [159]. In a recent study, 9 of 50 cases presented both 19 and 21 kDa peptides and they were mostly $129^{Met\ Val}$ heterozygotes [309]. It appears that accumulation of both peptides is a rule rather than an exception and that the putamen, medulla and the cerebellum are brain areas that accumulate those peptides most frequently. The same situation is seen in GSS 102^L in humans [281]. Two major phenotypes of this form of GSS exist: one that is characterized by the presence of spongiform change [156,216] and one without; both forms are characterized by the presence of amyloid plaques. From the GSS

phenotype with spongiform change, a 21 kDa PrP peptide was purified, but from the GSS form without only 8 kDa peptide was found. However, when Tg(PrP – P101L) mice were used in transmission experiments, only inoculum containing 21 kDa PrP peptide transmitted the disease. The inoculum containing 8 kDa did not transmit. Instead, large PrP deposits were seen without clinical signs [305].

Third, if an aggregate of PrP^d is a *bona fide* replicating prion, then the generation of new PrP^d in *in vitro* conversion experiments should generate new infectivity. Estimation of increased infectivity following the *in vitro* conversion reaction has been technically difficult because the amount of the *de novo* generated PrP^d is much less than the amount of original misfolded PrP^d used as a template for conversion [64]. However, this obstacle was overcome by using misfolded PrP^d seed from a species (hamster) which is non-infectious for the recipient species (mice) together with a chimeric normal protein (PrP^c) that can be propagated in both mice and hamsters [163]. The failure under these conditions to generate new PrP^d [64,163] was elegantly substantiated using transgenic mice. Transgenics with 9 extra octarepeats (PG14) developed "spontaneous" neurodegeneration and produced PrP^d that was resistant to PK, but their brains did not transmit the disease [76]. However, when PG14 transgenics were infected with scrapie, both PrP^d and infectivity were observed. Thus, PK-resistance of PrP^{PG14} and "spontaneous neurodegeneration" are not enough for the creation of infectivity – on the contrary, infectivity and "spontaneous neurodegeneration" have been clearly separated.

Finally, an *in vitro* protein misfolding cyclic amplification (PMCA) method has been developed that generates up to 30-fold increase in misfolded PrP^d, but initially no demonstrable increase in infectivity was reported [299]. PMCA is, like polymerase chain reaction (PCR), a cyclic process in which a minute quantity of PrP^d is incubated with PrP^c to generate more PrP^d. The seed of PrP^d must be sonicated before incubation with PrP^c. *De novo* generated PrP^d has the same biochemical properties as PrP^d derived from TSE-infected brains. The authors believed but did not prove that, following dilution of 10^{-55}, no TSE-infected-brain-derived molecule is present in the final sample, but they did not consider a caveat in which a cofactor molecule (such as an oligonucleotide) is copurified, even passively, with amplified PrP^d. Anyway, following inoculation of "newly formed PrP^d" into recipient hamsters, scrapie-like disease developed with an incubation time much longer than that reported for experiments with "normal" scrapie inoculum (177 vs. 106 days) [63]. What is more intriguing, when the sample was further diluted (10^{-10} to 10^{-20}), shortening of the incubation time was observed instead of the predicted increase of incubation time. Thus, either the newly formed PrP^d behaves as not "real" PrP^d (in other words, the insolubility and PK-resistance of the newly formed PrP^d are not sufficient to form a true "prion"), or an additional cofactor is necessary to complete the agent. Manuelidis [233] offers two explanations: either carry-over of a small amount of infectivity on sonication probes [368], or "intrinsically imperfect brain homogenate dilutions". Despite the fact that the results of Castilla et al. [63] could not be substantiated in subsequent experiments [27], they were hailed as final proof of the "prion hypothesis" – "the birth of the prion" [346]. Interestingly enough, Bieschke et al. [27] were able to reproduce amplification of PrP^d by PMCA, but not generation of infectivity. Those results may still be interpreted in two ways. Either generation of a small amount of infectivity is indeed the final proof of the prion hypothesis, or PMCA merely amplifies amyloid PrP^d in a seeded-nucleation process that is not enough to create "a prion". Thus, a search for a cofactor of the shell protein (PrP^d) to form the infectious unit is still needed.

Transgenic studies and the "species barrier" phenomenon

The most impressive and the most complex data suggesting either a close linkage between misfolded PrP^d and the infectious agent, or merely proving the necessity of the PrP^c-encoding gene for infection/replication of the agent, stem from the experiments using *transgenic* and *knock-out* mice technologies. It must be stressed that discrimination between these two alternatives is, at the present time, not possible. These experiments were developed because of the strong linkage of the occurrence of familial and sporadic forms of TSE with specific polymorphisms and mutations of the *Prn-p* gene (the mouse gene equivalent of the *PRNP* gene in humans).

The term "species barrier" (better "transmission barrier") [340] denotes a phenomenon in which the agent (prion) originating from one species is partially or entirely inhibited from infecting another species, measured either as a prolongation of the incubation period or inability to transmit the disease. For instance,

the 263K strain of the scrapie agent is pathogenic for hamsters but not for mice (mice may succumb to 263K but only after a very long incubation period of more than 700 days [193,195]. However, when transgenic mice were constructed with the hamster *PrP* gene (TgHaPrP) [289,310] they were found to be fully susceptible to the 263K strain of scrapie, and the incubation periods in different lines of transgenic animals were inversely proportional to the number of transgene copies and amount of PrP^c.

At this point, the "replication site hypothesis" formulated by Dickinson and Outram [96,99,100,107] is worth recalling. These investigators hypothesized that the scrapie agent is replicated via an interaction with a limited number of putative *replication sites* (agent receptors) which are heteromeric products of *Sinc*. The removal of replication sites, as in splenectomised [122] or genetically asplenic mice [95], prolongs the incubation period. Although the converse experiment (addition of replication sites) could not be accomplished at that time, it could be predicted that such an addition (via acquisition of extra copies of the *Sinc-PrP* transgene) would tend to *shorten* the incubation period, and such an effect was indeed observed in experiments using transgenic mice [289,310,351].

Although these studies did "not address the possibility of a putative second component within the prion, such as small nucleic acids" [311] they explicitly suggest that the interaction between misfolded PrP^d contained in the inoculum and heterologous host PrP^c is a major factor in the transmission barrier effect (the agent strain is also important because the species barrier is not the same for any pair of donor and recipient strains). It was hypothesised that such protein-protein interaction may result in PrP^d amplification (which thus merely *mimics* replication), not unlike that discovered for the mutant p53 oncoprotein interaction with its wild analogue.

The hypothesis of misfolding of PrP^c into PrP^d as underlying the pathogenesis of TSE [287] gained substantial support from studies of chimeric PrP proteins in transgenic mice [311]. When scrapie infected-murine neuroblastoma cell lines were transfected with chimeric *PrP* genes that consisted of various combinations of four different segments derived from either hamster or mouse *PrP* gene, only those chimeric proteins that were recognised as "murine" were converted into truncated proteinase K-resistant PrP27-30. Transgenic mice were also constructed with all these chimeric *PrP* transgenes as well as additional *MH2M* transgene that may represent an "intermediate" between mouse and hamster *PrP* genes. Analogous to *in vitro* studies, only those transgenics that harboured chimeric transgene recognized as hamster *PrP* were susceptible to the 263K strain of scrapie agent while those Tg mice that harboured transgene recognized as "murine" were resistant to this strain, and thus behaved like non-transgenic mice for which 263K strain of scrapie is non-pathogenic [335,336].

In contrast to Tg mice with hamster PrP that were susceptible to hamster scrapie, Tg mice constructed with human *PrP* ("humanized" Tg mice) became only partially susceptible to human CJD [336]. Construction on a null background somewhat increased this susceptibility, and human/hamster chimeric mice are highly susceptible to CJD. Analogously, "bovinized" mice became susceptible to BSE, while bovine/human transgenics were resistant [310]. These data were interpreted to mean that a host "protein X" interacts with a mouse PrP more readily than with human PrP, and thus blocks the interaction between human PrP contained in the inoculum and human PrP encoded by the transgene. Although an appropriate surface reaction site on the globular C-terminus of PrP has been mapped [185], the identity of "protein X" remains conjectural and eventually quietly died away. However, in "bovinized" transgenics obtained by the gene targeting method and inoculation with BSE, prolongation instead of shortening of the incubation period was observed. Thus, identity of donor and recipient PrP^s did not always lead to an abrogation of the transmission barrier [56].

In contrast, Collinge et al. reported that "humanized" Tg mice homozygous for valine (Val) at codon 129 are susceptible to both sporadic and iatrogenic CJD, irrespective of the status of the codon 129 genotype of the inoculum, but not for vCJD, which is homozygous for methionine (Met) at codon 129 [11,82]. Subsequent studies documented the general phenomenon that Met-homozygous Tg mice are more susceptible to Met-homozygous than Val-homozygous human CJD inocula [11]. These data strongly suggest that the congruence of the status of codon 129 in inoculum and the recipient mice underlies susceptibility to infection.

Surprisingly, a proportion of Met-homozygous Tg mice were found to develop subclinical infection following inoculation with vCJD [11]. Moreover, those mice that did not become sick showed two different phenotypes – one typical for vCJD characterized by the presence of florid plaques in the brain and a type 4 glycosylation pattern, and the other resembling sCJD with no florid plaques and a type 2 glycosylation pattern. The latter finding led to the suggestion that some patients diagnosed as sCJD

might in reality represent BSE infection, and explain the increased number of sCJD cases recently observed in Switzerland [137]. The pattern is more complex, however [11]. When Val-homozygous Tg mice were inoculated with vCJD inoculum, they propagated another glycosylation pattern – type 5 – and demonstrated a distinct neuropathological picture [11]. Furthermore, Met-Val-heterozygous Tg mice that overexpress the transgene 4–6-fold are susceptible to sporadic or iatrogenic CJD with all three codon 129 genotypes as well as vCJD [11]. The latter, however, showed no clinical signs and represent a subclinical infection. Those vCJD-infected heterozygous Tg mice readily demonstrate type 4 glycosylation pattern but a totally distinct neuropathological picture characterised by no florid plaques.

An even more complex pattern of altered susceptibility has been observed in transgenic mice with Leu at position 101 of the *Prn-p* gene (analogous to the 102^{Leu} mutation in GSS) obtained by gene targeting [12,13,56,228,229]. These mice are susceptible to the 263K strain of scrapie and to GSS, but are partially resistant to the 22A and 79A strains of scrapie and to vCJD, suggesting that interactions between host PrP^{c} and a given strain are strain-specific.

"Spontaneous" generation of infectivity

Several lines of transgenic mice that overexpress the mutant PrP develop "spontaneous" neurodegeneration, of which perhaps the most notorious are mice with a mutation at codon 101, homologous to the codon 102 mutation associated with GSS in man (*vide supra*; [170,171]). Brain tissues from these Tg mice overexpressing the transgene were originally reported to be devoid of PrP^{d} on Western blot [172] but were subsequently shown to contain PrP^{d}-immunoreactive plaques, and were reported to transmit spontaneous scrapie-like disease to 101L Tg mice but with a low copy number of the transgene, but not to wild-type mice [170]. However, these low-copy number transgenics (Tg196) also developed spontaneous degeneration after approximately 500 days.

In contrast, in a subsequent study of mice constructed by means of gene targeting (thus, without extra copies of the transgene), neither "spontaneous neurodegeneration" nor "disease transmission" was observed [228, 229], clearly suggesting that overexpression itself, and not the genetic construction of the prion, is responsible for spontaneous neurodegeneration. Furthermore, reported transmission from MoPrP(P101L) transgenics [171] seems to be only upregulation of the transgene to accelerate already incubating "spontaneous disease" [263]. Another experiment along those lines was reported recently by Legname and colleagues [207], in which recombinant PrP (rPrP) composed of residues 89-230 (recMoPrP(89-230)) obtained in E. coli was converted into β-fibrillar form [29] and injected into transgenic mice Tg9949 overexpressing the same sequence of PrP. Tg9949 mice became sick following injection of a preparation of β-fibrillar rPrP purified from E. coli after 380-660 days, but remained healthy after sham inoculation with PBS. There is a caveat here. Tg9949 mice were never observed (or at least reported) long enough (longer than 670 days) to exclude spontaneous neurodegeneration resulting from transgene overexpression as in the case of Tg mice overexpressing normal PrP [349]. In a further experiment Legname et al. [208] passaged "synthetic prions" to both transgenic and wild type FVB mice. The latter passage, however, produced two different isolates of different incubation times and neuropathological profiles. Again, it is not consistent with a single polypeptide, recPrP(89-230), primarily injected into the brains of tg mice. These recPrP(89-230) peptides produced 3 novel fragments: 12 kDa, 10 kDa, and 8 kDa [29, 30] and adopted β-fibrillary-rich conformations. Of interest, those recombinant peptides exist in two forms: the β-oligomer and the amyloid form [29].

Proof that PrP is crucial for the pathogenesis (but not necessarily for the infectious agent) came from knock-out mice ($PrP^{0/0}$) experiments [53,54]. These mice showed no clinical abnormalities during their normal life span [38,54] although hippocampal brain slices were reported to show impaired GABA receptor-mediated fast inhibition and long-term potentiation [83]. More importantly, these $PrP^{0/0}$ mice proved to be completely resistant to scrapie infection [53,287], and hemizygous mice ($Prp^{+/0}$) showed a prolonged incubation period. These results in effect "completed" the classical studies of asplenic mice [95] by reducing the number of peripheral and central replication sites to zero. Recently, cattle lacking PrP^{c} were also constructed and, like mice, they are resistant to BSE and healthy [290]. Although these experiments do not solve the problem of the nature of the scrapie agent, they do provide definitive proof that the *PrP* gene is indispensable in scrapie pathogenesis (and *eo ipso* in the development of clinical disease).

PrP gene mutations and TSE phenotypic expression

Specific changes in the *PrP* gene sequence are associated with different phenotypic expressions of the TSE.

As already mentioned, the scrapie incubation period both in mice and in sheep is controlled by *Prn-p* (classical names for this gene are *Sinc* and *SIP*, respectively, in mice and sheep). The early evidence for this linkage [97,98,100,105-107] was followed by the discovery of polymorphisms within the *Prn-p* gene which are linked to allelic differences affecting the length of the incubation period [57-59,350]. In mice, there are two major alleles, Prn-p^{a} and Prn-p^{b}, which differ at codon 108 and 189 [350]. The Prn-p^{a} allele encodes 108^{L} and 189^{T} while Prn-p^{b} allele encodes 108^{F} and 189^{V}. Using gene targeting, it was demonstrated that these polymorphisms control the incubation period in mouse scrapie [261]. Equivalent linkages were soon discovered in sheep [178,179]. For instance, Cheviot sheep with Val at codon 136 are susceptible to the SSBP/1 strain of scrapie, while those with Ala at this codon are not [177,179]. However, the linkage between haplotypes of PrP (*SIP*) gene in sheep and the susceptibility to other strains is extremely complex, and does not appear to depend on the presence of naturally occurring scrapie: sheep in Australia, where there is no scrapie, exhibit the same "susceptible" haplotypes as those elsewhere [178].

In man, the obvious candidate for such a linkage analysis was Gerstmann-Sträussler-Scheinker (GSS) disease and other familial forms of CJD [216,217,221, 244], and it was soon discovered that the expression of GSS is linked to a mutation (substitution of Pro with Leu) at codon 102 of the *PRNP* gene [167]. Several other mutations followed: two GSS families (from Indiana, USA and from Sweden), characterised by the occurrence of microtubule-associated protein (MAP)-τ-positive neurofibrillary tangles not unlike those of Alzheimer's disease [133,329], are linked to mutations at codon 198 (Phe to Ser) and 217 (Gln to Arg), respectively [115,168]. Familial CJD cases from all three known world clusters in Slovakia ("Oravske kuru") [148,150,258], Israel [147,169], and Chile [40] are linked to the codon 200 (Glu to Lys) mutation. Curiously, 200^{Lys} is associated not only with CJD in the "wandering Jew of the Diaspora" [21,44,127,142], as it has recently been discovered in a Japanese family [180]. Other CJD families are linked to mutations in codon 178 (Asp to Asn) [45,117,146, 205,264], as is fatal familial insomnia [149,260], which had been classified as "thalamic dementias" before its transmissibility was proved [81,333]. To date, more than 30 mutations (missense, nonsense and inserts) have been reported, and the number of new mutations may continue to grow [41-43,46,140,141,143,271].

The polymorphism at codon 129 merits special comment. Codon 129 encodes Met in 62.5% and Val in 37.5% of alleles in the normal Caucasian population [150,269,272]. However, in all forms of CJD, there is marked over-representation of homozygotes over heterozygotes [80,93,259,272]. In vCJD all the clinical cases are MM homozygotes. The codon 129 polymorphism may also exert a modifying effect on the phenotypic expression of a given *PRNP* mutation; for instance 129^{Val} is coupled with 198^{Ser} or 217^{Arg} in GSS [115,167], and 129^{Val} 178^{Asn} is linked to a CJD phenotype, whereas 129^{Met} 178^{Asn} is linked to fatal familial insomnia [145]. PrP proteins purified from familial CJD with 178^{Asn} and fatal familial insomnia are different, and these differences are probably conformational [260].

Three lines of transgenic mice (MM, MV, and VV) were produced by gene targeting methods and inoculated with vCJD [56]. All these lines were susceptible to vCJD, with the shortest incubation period in MM mice and the longest in VV mice. These results suggest that all individuals may be susceptible to vCJD, but vCJD in VV homozygotes may yet to be observed, and thus epidemics of vCJD may be yet to occur.

The situation with kuru is particularly interesting. The practice of endocannibalism underlying the kuru epidemic created a selective force on the prion protein genotype [4,39]. As in CJD, homozygosity at codon 129 (129$^{Met\ Met}$ or 129$^{Val\ Val}$) is overrepresented in kuru [68,144,213]. However, Mead et al. [248] found that among Fore women over fifty years of age, there is a remarkable overrepresentation of heterozygosity (129$^{Met\ Val}$) at codon 129, which is consistent with the interpretation that 129$^{Met\ Val}$ makes an individual resistant to TSE agents and that such resistance was selected by cannibalistic rites. Because of this 129$^{Met\ Val}$ heterozygote advantage, it has been suggested that the heterozygous genotype at codon 129 has been sustained by a widespread ancient practice of human cannibalism [91,237].

The existence of familial TSEs (fTSEs – namely fCJD, GSS and FFI) caused by mutations in the *PRNP* gene may also be interpreted two ways. According to the protein-only hypothesis, any given mutation may change the energy barrier for a misfolding of PrP^{c} into PrP^{d} and thus cause disease. According to the virus theory, the mutation merely changes the susceptibility to a ubiquitous virus [66,231]. There are precedents for such a mechanism: mutations responsible for sickle cell disease or thalassaemias increase susceptibility to B19 parvovirus.

PrP as amyloid: the concept of transmissible cerebral amyloidosis

Amyloid plaque has long been recognised as a hallmark of the neuropathology of some TSEs, especially kuru and GSS [197] – indeed kuru was nicknamed the "galloping senescence of the juvenile" [127] – and it is possible that kuru originated from the core of GSS incidentally cannibalized (Liberski – Budka, personal communication). More than three decades later, it was established that the amyloid plaque of TSE was mostly composed of misfolded PrP^{d}. As a result, Gajdusek suggested calling PrP 27-30 "scrapie amyloid" [126,127,301-304] and proposed that the conversion of normal into misfolded protein may become "autocatalytic when the baby crystals continue the pattern-determining nucleation process" [125]. Fibrillar structures isolated from TSE-affected brains (SAF or prion rods) are morphologically very similar to, but distinguishable from, many other amyloid fibrils when visualised by negative-staining electron microscopy [252,253,288]; however, due to technical problems associated with protein insolubility, direct evidence for a cross β-pleated secondary structure of PrP^{d} was obtained only within the last decade. Using infrared spectroscopy, which correlates the infrared spectrum with the secondary structure of proteins and does not require the protein to be in solution, Caughey et al. [67] showed that PrP 27-30 contains a high proportion of β-pleated sheet, which was later quantified by Gasset et al. [130, 131], who estimated the proportion β-conformation to be approximately 50% of the entire structure. Denaturation by either SDS or high pH reduces both scrapie infectivity and β-pleated content [47].

The predicted conformation of PrP^{c} suggested four α-helices and two β-strands [173], whereas the conformation of PrP^{Sc} is largely β-pleated. However, the conformation of synthetic peptides derived from regions of predicted secondary structures of PrP had a protean nature. Gasset et al. [131] demonstrated that H1 (the first helix) *in vitro* is in a cross-β-pleated conformation; H2 may form either α-helix or β-strand or a β-*turn*; while H3 and H4 have a β-strand structure. Furthermore, Heller et al. [162] showed that H1 may have either an α-helix or β-strand conformation. Collectively, these studies indicate that PrP is able to form either α-helices or β-pleated sheets and conversion from α-helix into a β-sheet may underlie the formation of prions. Indeed, using the hamster recombinant protein (r)PrP (residues 90-231) Mehlhorn et al. [251] demonstrated that the same peptide may form stable α-helix or β-sheets with several intermediates. All these data point to an inherent propensity of PrP to exist in different conformations, and Jackson et al. [182] showed that even a truncated form of PrP containing only residues 91-231 may adopt multiple conformations dependent on pH, redox conditions and denaturant concentration.

Using NMR spectroscopy, Riek et al. [28,291,292] found the secondary structure of mouse PrP (residues 121-231) in solution to consist of three α-helices and two antiparallel β-strands at the globular C-terminus, with a largely unstructured (flexible) N-terminus. Similar conformations characterized a longer recombinant PrP (residues 23-231) fragment [292] as well as conformations of hamster [183,223], bovine [224], and human [366] PrP^{c}. A comparison between human, mouse and bovine PrP demonstrated that human and bovine PrP^{s} exhibit virtually identical conformations [224], and because species susceptibility is strongly influenced by PrP^{c} conformational similarity, humans may be presumed to be a species "of choice" for BSE infection.

The level of glycosylation may affect the structure of PrP. As already mentioned, both the normal and misfolded proteins possess two N-glycosylation sites at N^{181} and N^{197}, and an O-glycosylation is also possible through Ser-132 and Ser-135 [73]. Studies of a synthetic PrP 109–144 [73] demonstrated that the addition of αGalNac at Ser135 suppressed spontaneous *in vitro* fibril formation, whereas addition of 132-αGalNac at the same site stabilized the β-structure and increased fibril formation.

Further complexity of PrP conformational changes was evidenced by observations of two-dimensional crystal preparations of PrP 27-30 and its analogue $PrP^{d}106$ ("miniprion"), the shortest truncated form of PrP which still retains infectious properties [354]. Analysis of these two-dimensional lattices suggested the existence of a new form of PrP^{Sc} – the so-called β-helix. Furthermore, CD and NMR studies of PrP peptide 142-166 spanning helix 1 and β-strand 2 revealed an additional β-hairpin structure [200].

It is now widely accepted that many structurally diverse proteins can misfold and cause so-called "conformational diseases", including the most common neurodegenerations, Alzheimer's disease and Parkinson's disease [313]. The conversion of largely α-helical or random coil proteins into cross-β-pleated sheet conformations that form first oligomers and then fibrils underlies these disorders. However, this α- to β-structure transition seems

to be a generic propensity of all globular proteins, not only those involved in neurodegenerations [29]. Metaphorically, all these neurodegenerations are "infectious" in the sense that misfolded β-sheeted conformers are formed in a nucleation process in which pre-formed metastable oligomer acts as a seed (a nucleus) to convert a normal into an abnormal protein. However, in none but TSE has infectivity in a microbiological sense ever been observed, and even in TSE the formation of misfolded protein is not necessarily accompanied by the generation of infectivity *de novo*. Surprisingly, GSS P102L inoculated into Tg PrP(P101L) mice produced no clinical disease bur robust PrP^{d} deposition. That may suggest that certain "prion diseases" are not TSEs but just "proteinopathies" caused by accumulation of abnormally misfolded PrP^{d} [13]. The presence of a massive amount of PrP-amyloid and no infectivity casts doubts on whether TSEs are really infectious amyloidoses [281]. Additionally, when PrP^{Sc} was solubilized with the chemical chaperone dimethyl sulfoxide (Me_2SO) and then Me_2SO was removed, solubilized PrP reformed into oligomers with only a minimal amount of infectivity [314]. The misfolding of PrP may yet prove to be an epiphenomenon secondary to infection with a still unknown infectious agent [215,232].

If, on the other hand, the purely proteinaceous character of the replicating unit of TSE infectivity is ultimately found to be correct, the critical issues become 1) the mechanism by which a misfolded PrP template induces normal protein molecules to adopt the same pathologically misfolded conformation, and 2) the intracellular conditions that are responsible for strain differences in these molecules. The search goes on.

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