

Prion Diseases: from transmission experiments to structural biology – still searching for the cause

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This paper is dedicated to Dr D. Carleton Gajdusek on the occasion of his 80th birthday

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative disorders that include kuru [106], Creutzfeldt-Jakob disease (CJD) [112], Gerstmann-Sträussler-Scheinker (GSS) disease [188], and fatal familial insomnia [173, 192, 193] in man, natural scrapie in sheep, goats [67–70] and mufllons [263], transmissible mink encephalopathy in ranch-reared mink [42], chronic wasting disease of mule deer and elk in the USA [130, 170, 258–260], bovine spongiform encephalopathy or “mad cow disease” [252] and its analogues in several exotic species of antelopes [71, 97, 152, 162] and wild felids in Zoological gardens [261] and feline spongiform encephalopathy in domestic cats [264].

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”. Despite wide acceptance for the prion theory, these names still reflect different views about the true 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 protein (PrP^{res}, PrP^{Sc}; Sc, from scrapie or PrP^d; d, from disease), use the term “prion” [216, 218, 219, 221]; hence the term “prion diseases”.

The “virino” hypothesis suggests that the pathogen is a molecular chimera composed of a still-to-be-discovered nucleic acid and a shell-protein that is host-encoded (perhaps PrP^d) [155–157]. The virus hypothesis simply suggests that the pathogen is a yet-to-be-identified unconventional virus [93, 94, 181]. The “unified theory” of Weissmann [250, 251], 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 folle”, “le tremblante” “la prurigo lombarie” 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*) [9] and, as a paragraph in the *General View of the Agriculture of Wiltshire* published by Thomas Davies in 1811 [198]. In 1848, Roche-Lubin [225] claimed that scrapie is caused by sexual excesses of rams or, alternatively, by thunderstorms. M’Gowan [198] himself suggested *sarcosporidium* as the causative agent.

The first interpretation of scrapie (*tremblante*) as a virus disease is credited to the French veterinarian Bessnot in 1899 [17], and the transmissible nature of TSEs was proven in 1936 by the seminal experiments of Cuille and Chelle [65–70]. The contention that scrapie is an infectious disease caused by a filterable agent was ac-

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cepted with scepticism until 1938, when W.S. Gordon, 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 [127, 214]. Ironically, the infectious nature of the scrapie agent had already been unintentionally confirmed in 1935, when some 7% of 18.000 sheep vaccinated against Louping ill vaccine developed scrapie [126, 128]. The vaccine had been produced from sheep brains and also showed that scrapie infectivity could survive 0.35% of formalin for more than 3 months. World War II interrupted scrapie research continued by D.R. Wilson [262], 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 [52–55] and from sheep to mice by Morris and Gajdusek [204], 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 [7, 111, 143–145] or a subvirus (not well envisaged) linked to a membrane with a „linkage substance” [1, 2], a viroid [90–92, 174, 175, 183, 185–187], a spiroplasma [14] (Fig. 1), or a retrovirus-like element [4–6, 178, 238, 244].

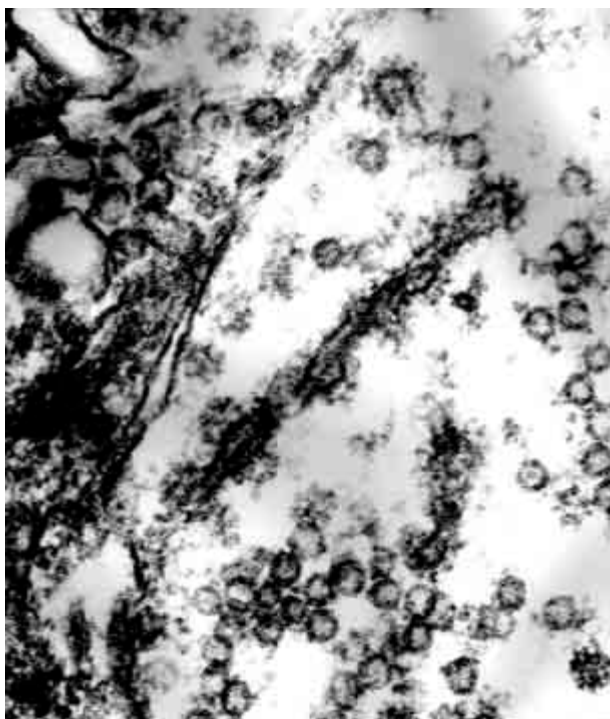


Figure 1. “Spiroplasma-like inclusion” represents in reality an array of microtubules associated with coated vesicles. Scrapie-infected hamster brain, original magnification, $\times 85.000$.

Suffice it to say that none of these hypotheses could be substantiated despite an exhaustive use of all methods of both classical and molecular virology.

The first TSE in humans was kuru discovered by Gajdusek and Zigas [107; see also Liberski and Brown, this volume, pp 3–14]. The elucidation of Kuru opened a new field in human medicine and initiated 40 years of research, which has contributed enormously to our understanding of neurodegenerative disorders of the central nervous system including Alzheimer’s disease [104, 166, 169]. Kuru was transmitted to chimpanzees in 1965 [106], and was quickly followed by transmission of Creutzfeldt-Jakob disease [112] and GSS [188]. The list was apparently closed by the recent transmission of fatal familial insomnia [62, 245].

In 1987, Gerald A.H. Wells and his colleagues described a cow with a novel form of TSE [252] (parenthetically, the first case of BSE had actually been observed in a Nyala by Jeffrey *et al.* [152]). The BSE epidemic reached a climax in 1992 with more than 37.000 cases, and their number then steadily declined to 612 in 2003. In 2004, there were 4 BSE cases found by passive and 3 by active surveillance but no official OIE numbers are available for the UK (see, Bradley, this volume). However, in 1996 Will *et al.* [256] reported a new variant of CJD (vCJD), suggesting on epidemiological evidence to be due to BSE infection, and subsequently substantiated by laboratory studies [39, 63, 135, 235]. Although the number of vCJD cases is decreasing (only 1 case of vCJD has been identified since January 2004) 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 changed 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 [101]. 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. PrP 27–30 is a proteolytic cleavage product of PrP^d [22, 189].

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 [13, 57, 206, 239; see Bratosiewicz-Wąsik *et al.* this volume, pp 33–46].

The gene is ubiquitous; it has been cloned in numerous mammalian species, included marsupials, and there are analogues of this gene in birds [102, 131], reptiles [237], amphibians [242], and fish [207]; those in *Drosophila* and nematodes appeared to be cloning artefacts [255]. 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 [22, 189], which led to the conclusion that PrP is a 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 [215, 217]. 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 a decade earlier by Gibbons and Hunter [111], Griffith [129], Levine [168], and who all developed the earlier suggestion of Alper and her co-workers [8], based on irradiation studies, that scrapie agent was devoid of disease-specific nucleic acid. While the theoretical approach of Alper *et al.*, was criticised by Rohwer [226, 227], several investigators had found previously that scrapie infectivity was sensitive to proteolytic digestion [59, 199].

Like many amyloid proteins, PrP^{27–30} is a proteolytic cleavage product of a precursor protein, PrP^{33–35^d}. However, PrP^{33–35^d} is not the *primary* product of the cellular gene. It has an amino acid sequence, glycosyl-inositol phospholipid anchor and posttranslational modifications (like glycosylation and the attachment of GPI, glycopospholipid inositol anchor) identical to those of PrP^{33–35^c} but strikingly different physico-chemical features [240, 241]; in particular, PrP^c is completely degraded by a 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) [195, 196], or prion rods (Fig. 2) [221]. To become PrP^d, PrP^c must first be transported to the cell surface and then through the endosomal-lysosomal pathway [23, 190].

PrP has several interesting features. As already mentioned, it is a glycoprotein with two Asn-glycosylation sites; thus, PrP may exist as deglycosylated, monoglycosylated and di-glycosylated isoforms of different electrophoretic mobilities and glycoforms [63] and the various combinations of glycosylation and codon 129 genotype (see later) correlate to some degree with the phenotypic expression of TSE. In particular, a distinctive glycosylation pattern is uniquely present in both BSE and vCJD (Fig. 3) [63, 135]. Although glycosylation patterns breed true – *i.e.* they are retained in passage [63]

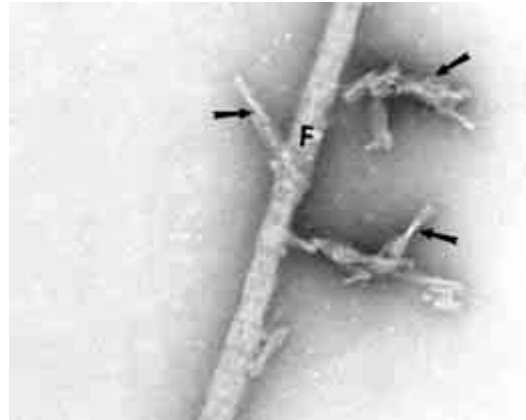


Figure 2. Fibrillar aggregates of PrP as visualized by negative-staining electron microscopy. Original magnification, × 50,000.

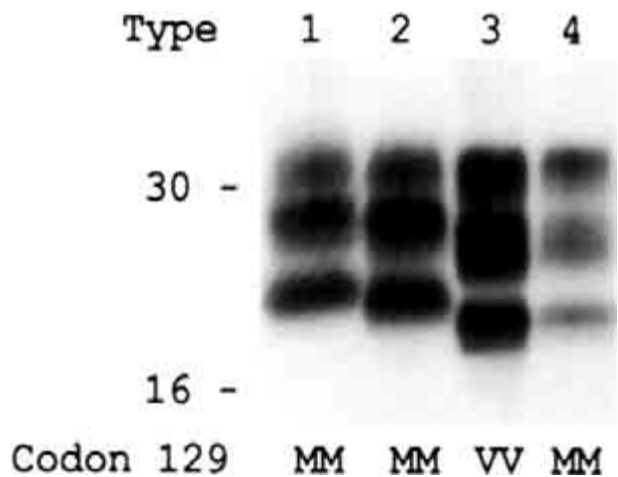


Figure 3. Glycotypes 1–4 according to the Collinge’s classification. The 4th glycotype is unique for both vCJD and BSE. Courtesy of Prof. John Collinge, London.

– interconversions may occur in the presence of metal ions [63, 249], 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 [15].

STRAINS OF THE AGENT – STILL THE STRONGEST ARGUMENT AGAINST THE PROTEIN-ONLY HYPOTHESIS

From the early work of Alan G. Dickinson and his collaborators [84, 86–89, 99] it was known that the incubation period of scrapie was tightly linked to the gene designated *Sinc* (in mice; from **s**crapie **i**ncubation) and *SIP* (in sheep; from **s**horter **i**ncubation **p**eriod); Parry [213] even proposed that scrapie was a primarily genetic disorder that was only secondarily transmissible (agitated discussion between Dickinson and Parry dominated the field in the 1960’s) [85]. The discovery

of *Sinc* was instrumental in supporting the notion that the scrapie agent has an independent genome [36]. It was subsequently shown that *Sinc* (*SIP*) is identical with the gene encoding for PrP [38] and that *Sinc*^{s7} is congruent with *Prn-p*^a while *Sinc*^{p7} is congruent with *Prn-pb* [203]. Furthermore, *Prn-p*^a (*Sinc*^{p7}) and *Prn-p*^b (*Sinc*^{s7}) differ by two aminoacids in position 109 and 189 [253]. 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 structural biology of PrP have challenged this statement.

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.* semiquantitative estimation of spongiform change in terms of the brain topography [35, 38, 88]. 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 new characteristics that are stable in subsequent passages. Such changes would be consistent with the effects of mutations in a (as yet unidentified) disease-specific nucleic acid.

Approximately 20 strains of scrapie agent have been isolated from sheep and goats affected with clinical scrapie [47, 75]. 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 [46, 75]. Some sources of sheep scrapie are not transmissible to mice, for example the CH 1641 isolate [98]; 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 exhibit a short incubation period when passaged through *Sinc*^{s7} (*Prn-p*^A) mice (s for short; for example C57Bl mice) and a long incubation period when passaged through *Sinc*^{p7} (*Prn-p*^B) mice (p for prolonged; for example VM mice). The 22A group exhibit exactly the opposite characteristics: short incubation period in *Sinc*^{p7} mice and long incubation period in *Sinc*^{s7} mice [76, 78–80]. 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* [47, 146, 158, 203]. All recent scrapie isolates differ from the BSE strain, but the spectrum of scrapie strains may have changed over the last 20 years [35].

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 [60, 159–161]. One of the best known exam-

ples of the isolation of a mutant strain with completely different characteristics from the original isolate is the isolation of the 263K (the same as 237sc) strain of scrapie agent [159, 160]. Preservation of strain properties following passage through hosts of different *Prn-p* sequences strongly argues against proteina-only nature of the scrapie agent. Two sets of experiments may be cited in support of this viewpoint.

First, strains of scrapie agent may undergo phenotypic changes in incubation period, lesion profile, and the presence and amount of PrP amyloid deposits, which are compatible with mutations of “conventional” pathogens [36]. Three classes of strain stability have been established [36]. Class I stability strains (ME7, 22C) possess stable characteristics irrespective of the *Sinc* (s7 or p7) (*Prn-p*^A or *B Prn-p*^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 [37], 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 [36].

“Class III breakdown” was defined as a “*sudden shortening of an incubation period, in the course of single mouse passage, accompanied by a marked change in neuropathology*” [36]. This usually occurred at some point between the primary and the 7th 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 production of amyloid plaques. All these characteristics are reminiscent of ME7 and it is highly probable that 7D is actually the same as the ME7 strain of scrapie agent. The ensemble of these data favors 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 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 [253]. Often the relative incubation periods of two given strains can be reversed on changing the mouse strain [46].

The most notorious example of a strain of TSE that can be isolated from a different host is the strain that caused vCJD and exhibits the same operational characteristics (“BSE signature”) as the causative strain of BSE [39, 135, 235]. The BSE/vCJD strain is characterised by a distinctive glycosylation pattern [63] that is also seen in the brains of sheep infected with the CH1641 strain of scrapie [136]; however, the vCJD/BSE strain is readily transmissible to mice whereas the CH1641 strain is not [98]. The existence of the CH1641 strain of scrapie underlines the fact that glycosylation patterns may not tell the whole story about strain characteristics.

A second, independent, set of experiments focused on the phenomenon of agent “competition”: different strains of scrapie agent can exhibit competition when inoculated at different times, either intracerebrally (*i.c.*) [80] or peripherally [79]. 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 the 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 the 22C (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 [161] 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 of which are encoded by *Sinc* [76, 78–80]. 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 [180]. 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 [179].

The existence of strains may be explained by a strain-specific oligonucleotide or a ubiquitous virus; however,

neither one nor the other have ever been found despite repeated attempts to detect disease-specific nucleic acids [6, 154, 197]. It must be stressed, however, that even in the most highly purified fractions containing PrP^{Sc}, nucleic acids as long as 5000 bp can readily be found [179].

Another relevant observation was made following a 1985 outbreak of transmissible mink encephalopathy (TME) in Stetsonville, USA [184]. 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 strain differ [19, 20]. Using an *in vitro* PrP^c to PrP^d conversion reaction [48, 164] 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 [18]. This experiment suggested that 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 on the Western blot.

The banding pattern resulting from the conformation of PrP that determines the cleavage site for PK is also strain-specific, as was subsequently shown by experimental transmission of FFI and CJD to transgenic mice harbouring the chimeric mouse/human transgene [246, 247]. Following deglycosylation, the molecular weight of PrP^{FFI} is 19 kDa while that of PrP^{CJD} 21 kDa [202, 211], and the size of PrP^d (either 19 or 21 kDa) was retained during passage in Tg mice [246]. These observations were extended by Collinge *et al.* [63] who showed that the glycosylation pattern of PrP^d also “breeds-true” and indeed the preservation of this glyco-type 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 [135, 235]. As recently summarized by Caughey [48], these experiments suggest that “different PrP^d strains” can impose their different conformations on a single species of unglycosylated PrP^c. Caughey [48] further suggested that PrP^{Sc} may operate on different pools of PrP^c. The latter may reflect different cell (neuronal) populations in which these PrP^c molecules reside, 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 [73].

These experiments have also 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 [51], 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 [75], and only two PrP^d size species (21 kDa type 1 and 19 kDa type 2) have been isolated from human TSE phenotypes. Thus, if the existence of strains is reflected by the size of PrP^d peptides, all of the diverse phenotypes of CJD, GSS and FFI phenotypes are caused by just two strains, which seems highly unlikely. Even the more complicated genotype schema of Collinge *et al.* [60, 63], 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 [212]; 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), two glycosylation patterns have recently been observed [132]. Although multiple strains have been isolated from sheep brains, it has never been shown that these, in turn, are linked to multiple PrP conformers.

Third, if an aggregate of PrP^d is a *bona fide* replicating prion, then the generation of new PrP 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 [48]. 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 [246, 247, 248]. The failure under these conditions to generate new PrP^d [48, 134], 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 [58]. 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 – to 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 no

demonstrable increase in infectivity [228]. Thus, either the newly formed PrP^d is not “true” PrP^d (in other words, the insolubility and PK-resistance of the newly formed PrP^d are not sufficient to form true a “prion”), or an additional cofactor is necessary to complete the agent.

TRANSGENIC STUDIES AND THE PHENOMENON OF ‘SPECIES BARRIER’

The most impressive data suggesting either a close linkage between misfolded PrP and the infectious agent, or at least proving the necessity of the 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 the 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” denotes a phenomenon in which 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 disease. For instance, the 263K strain of scrapie agent is pathogenic for hamsters but not for mice (mice may succumb to 263K but only after very long incubation period of more than 700 days [159, 161]). However, when transgenic mice were constructed with the hamster *PrP* gene (TgHaPrP) [222, 234], 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 [222].

At this point, the “replication site hypothesis” formulated by Dickinson and Outram [76, 78, 79] is worth recalling. These investigators hypothesized that the scrapie agent is replicated in a limited number of putative *replication sites* (agent receptors), which are heteromeric products of *Sinc*. The removal of replication sites, as in splenectomized [100] or genetically asplenic mice [77] 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 *PrP* transgene) would tend to *shorten* the incubation period, and such an effect was indeed observed in experiments using transgenic mice [222, 234, 254].

Although these studies did “*not address the possibility of a putative second component within the prion, such as small nucleic acids*” [234], they explicitly sug-

gest that the interaction between misfolded PrP^d contained in the inoculum and heterologous host PrP^c is a major factor in the species 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 [219] gained substantial support from studies of chimeric PrP proteins in transgenic mice [233, 234]. 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.

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 [248]. Construction on a null background somewhat increased this susceptibility, and human/hamster chimeric mice are highly susceptible to CJD. 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 [153], the identity of “protein X” remains conjectural.

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 [10, 63]. Subsequent studies documented the general phenomenon that Met-homozygous Tg mice are more susceptible to Met-ho-

mozygous than Val-homozygous human CJD inocula [10]. 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 [10]. Moreover, those mice that did 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 [113].

An even more complex pattern of altered susceptibility has been observed in transgenic mice with Leu at position 101 of *Prn-p* gene (analogous for 102^{Leu} mutation in GSS) [11, 12, 176]. 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 and a given strain are strain-specific.

Several lines of transgenic mice that overexpress the mutant PrP develop “spontaneous” neurodegeneration, of which perhaps the best studied are mice with a mutation at codon 101, analogous to the codon 102 mutation associated with GSS in man [*vide infra*; 137, 139]. Brain tissues from these Tg mice overexpressing the transgene were originally reported to be devoid of PrP^d on Western blot [141], 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 [141].

However, in subsequent study of mice constructed by means of reciprocal recombination (thus, without extra copies of the transgene), neither “spontaneous neurodegeneration” nor “disease transmission” was observed [176, 177], clearly suggesting that overexpression itself, and not the genetic construction of the prion, is responsible for spontaneous neurodegeneration.

Proof that PrP is crucial for the pathogenesis, (but not necessarily the infectious agent) came from knockout mice (PrP^{0/0}) experiments [41]. These mice showed no clinical abnormalities during their normal life span [24] although hippocampal brain slices were reported to show impaired GABA receptor-mediated fast inhibition and long-term potentiation [64]. More importantly, these PrP^{0/0} mice proved to be completely resistant to scrapie infection [40, 219], and hemizygous mice (*Prp*⁺⁰)

showed a prolonged incubation period. These results in effect “completed” the classical studies of asplenic mice [77] by reducing the number of peripheral and central replication sites to zero. Although these experiments did not solve the problem of the nature of the scrapie agent, they did 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 [28–31, 33, 115–118, 119]. As already mentioned, the scrapie incubation period both in mice and in sheep is controlled by *Prn-p* (classical names for this gene is *Sinc* and *SIP*, respectively, in mice and sheep). The early evidence for this linkage [81–84, 86, 88] was followed by the discovery of polymorphisms within the *Prn-p* gene that are linked to allelic differences affecting the length of the incubation period [43–45, 253]. Equivalent linkages were soon discovered in sheep [147, 148]. 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 [146, 148]. 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 [147].

In man, the obvious candidate for such a linkage analysis was Gerstmann-Sträussler-Scheinker (GSS) disease and other familial forms of CJD [188], and it was soon discovered that the occurrence of GSS is linked to a mutation (substitution of Pro with Leu) at codon 102 of the *PRNP* gene [137]. Several other mutations followed: two GSS families (from Indiana, USA and from Sweden), characterised by the occurrence of microtubule-associated protein (MAP)-t-positive neurofibrillary tangles not unlike of those of Alzheimer’s disease [110, 243], are linked to mutations at the codon 198 (Phe to Ser) and 217 (Gln to Arg), respectively [95, 138]. Familial CJD cases from all three known world clusters in Slovakia (“Oravske kuru”) [123, 125, 200], Israel [122, 140] and Chile [27] are linked to the codon 200 (Glu to Lys) mutation. Curiously, 200^{Lys} is associated not only with CJD in “wandering Jew of the Diaspora” [16, 31, 105, 118], as it has recently been discovered in a Japanese family [149]. Other CJD families are linked to mutations in codon 178 (Asp to Asn) [16, 32, 96, 121, 167, 205], as is fatal familial insomnia [124, 202], that was earlier

Table 1.

Mutation	Disease phenotype
Octa-repeat insertions of 24, 48, 96, 120, 144, 168, 192, or 216 base pairs (between codons 51 and 91)	CJD, GSS, or atypical dementias
P102L (Pro Leu)	GSS: classical ataxic form
P105L (Pro Leu)	GSS: spastic paraparesis variant
A117V (Ala Val)	GSS: pseudobulbar variant
G131V (Gly Val)	GSS: classical ataxic form
Y145* (Tyr Stop)	Alzheimer-like dementia
D178N (Asp Asn)	CJD (129V on mutant allele)
D178N (Asp Asn)	FFI (129M on mutant allele)
V180I (Val Ile)	CJD
T183A (Thr Ala)	Alzheimer-like dementia
H187R (His Arg)	GSS: classical ataxic form
F198S (Phe Ser)	GSS with neurofibrillary tangles
E200K (Glu Lys)	CJD
D202N (Asp Asn)	GSS with neurofibrillary tangles
V203I (Val Ile)	CJD
R208H (Arg His)	CJD
V210I (Val Ile)	CJD
E211Q (Glu Gln)	CJD
Q212P (Gln Pro)	GSS with Lewy bodies
E217R (Glu Arg)	GSS with neurofibrillary tangles
M232R (Met Arg)	CJD
M232T (Met Thr)	GSS
Polymorphisms	
M129V (Met Val)	Phenotypic determinant
Octa-repeat (24 bp) deletion	Atypical dementias (?)

classified as “thalamic dementias” [62, 245]. To date, more than 30 mutations (missense, nonsense and inserts) have been reported, and the number of new mutations may continue to grow [28–30, 33, 116, 117, 119, 209] (Table 1).

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 [125, 208, 210]. However, in all forms of CJD, there is marked over-representation of homozygotes over heterozygotes [27, 61, 74, 201, 210]. The codon 129 polymorphism may also exert a modifying effect on the phenotypic expression of a given *PRNP* mutation, for in-

stance – 129^{Val} is coupled with 198^{Ser} or 217^{Arg} in GSS [95, 137], and 129^{Val} 178^{Asn} is linked to a CJD phenotype, whereas 129^{Met} 178^{Asn} is linked to fatal familial insomnia [120]. PrP proteins purified from familial CJD with 178^{Asn} and fatal familial insomnia are different, and these differences are probably conformational [202].

The situation in kuru is particularly interesting. The practice of cannibalism underlying the kuru epidemic created a selective force on the prion protein genotype [3, 25]. As in CJD, homozygosity at codon 129 (129^{Met/Met} or 129^{Val/Val}) is overrepresented in kuru [114]. However, Mead *et al.* [191] 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^{Val/Met} makes an individual resistant to TSE agents and that such a 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 [72, 182].

The existence of familial TSEs (CJD, GSS and FFI) caused by mutations in the *PRNP* gene may also be dually interpreted. 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 a virus theory, the mutation merely changes a susceptibility to an ubiquitous virus [49, 179]. 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

The amyloid plaque has long been recognised as a hallmark of the neuropathology of some TSEs, especially GSS and kuru [163] – indeed kuru was “nicknamed” the “galloping senescence of the juvenile” [105]. 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” [104, 105, 229–232] and proposed that the conversion of normal into misfolded protein may become “*autocatalytic when the baby crystals continue the pattern-determining nucleation process*” [103]. Fibrillar structures isolated from TSE-affected brains (SAF or prion rods) are morphologically very similar to but distinguishable from other amyloid fibrils when visualised by negative-staining electron microscopy [195, 196, 221]; 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 for the protein to be in solution, Caughey *et al.*, [50] showed that PrP 27–30 contains a high proportion of β -pleated sheet, which was later quantified by Gasset *et al.*, [108, 109], 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 [34].

The predicted conformation of PrP^c suggested four α -helices and 3 β -strands [142] 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.* [109] 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.* [133] 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 hamster recombinant protein, (r) PrP (90–231), Mehlhorn *et al.* [194] demonstrated that the same peptide may form stable α -helix or β -sheets with several intermediates. All these data pointed to an inherent propensity of PrP to exist in different conformations, and Jackson *et al.* [150] 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.* [21, 223, 224] found the secondary structure of mouse PrP (121–231) in solution to consist of three α -helices and 2 antiparallel β -strands at the globular C-terminus (Fig. 4), with a largely unstructured (flexible) N-terminus. Similar conformations characterized a longer recombinant PrP (23–231) fragment [224] as well as conformations of hamster [151, 171], bovine [172] and human [265] PrP^c. A comparison between human, mouse and bovine PrP demonstrated that human and bovine PrPs exhibit virtually identical conformations [172], and because species susceptibility is strongly influenced by PrP conformational similarity, humans may be presumed to be species „of choice” for BSE infection.

The level of glycosylation may affect the structure of PrP. Both the normal and misfolded proteins possess two N-glycosylation sites at N¹⁸¹ and N¹⁹⁷, and an O-glycosylation is also possible through Ser-132 and Ser-135 [56]. Studies of a synthetic PrP 109–144 [56] demon-

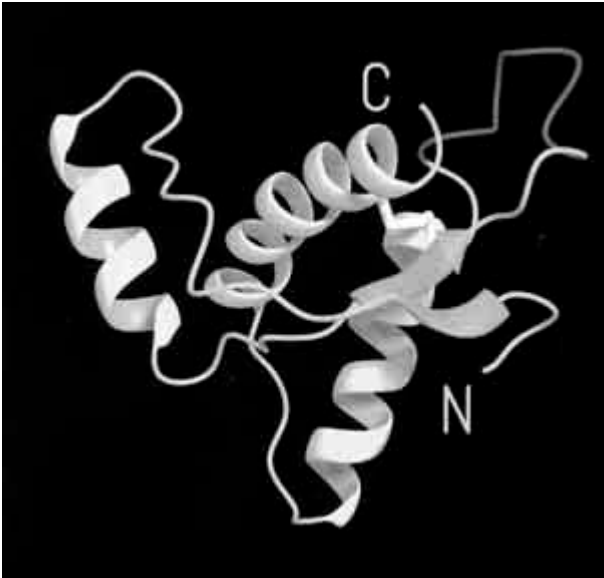


Figure 4. Secondary structure of recombinant PrP^c(121–231) in solution to consist of three α -helices and 2 antiparallel β -strands at the globular C-terminus.

strated that the addition of α -GalNac at Ser135 suppressed spontaneous *in vitro* fibril formation, whereas addition of 132- α GalNac at the same site stabilized the β -structure and increased fibril formation.

Further complexity of PrP conformational changes was evidenced by observations of 2-dimensional crystals preparations of PrP 27–30 and its analogue PrP^d106 (“miniprion”), the shortest truncated form of PrP that still retains infectious properties [257]. Analysis of these 2-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 [165].

It is now widely accepted that structurally diverse proteins can misfold and cause so called “conformational diseases” including the most common neurodegenerations, Alzheimer’s disease and Parkinson’s disease [236]. The conversion of largely α -helical or random coil proteins into cross- β -pleated sheet conformations that form 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. Metaphorically, all these neurodegenerations are “infectious” in the sense that misfolded β -sheeted conformers 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 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*. Thus, 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. The search goes on.

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