**Review** paper

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# Historical overview of prion diseases: a view from afar

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**Abstract**

*The transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of neurodegenerative disorders which include kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome, and fatal familial insomnia in men, natural scrapie in sheep, goats and mufflons, transmissible mink encephalopathy in ranch-reared mink, chronic wasting disease of mule deer and elk, bovine spongiform encephalopathy or "mad cow disease" and its analogues in several exotic species of antelopes and wild felids in zoological gardens, and feline spongiform encephalopathy in domestic cats.*

*This short review summarizes the history of the research to find the nature of the scrapie agent, especially as I have witnessed it unfolding before my eyes. I review the historical background of TSEs starting from the first description of scrapie in 1732. In 1957, the first prion disease in humans, kuru was described and its transmissibility was demonstrated in 1965 by seminal work of Gajdusek, Gibbs and colleagues, followed by transmission of CJD and then, GSS. In 1982, Stanley B. Prusiner formulated "prion hypothesis" which has dominated the field for the last 30 years. This theory had been recently extended to cover other neurodegenerations which are caused by misfolded proteins; these disease are called prionoids.*

***Key words:*** *prion, transmissible spongiform encephalopathies, kuru, scrapie.*

## Introduction

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of neurodegenerative disorders which include kuru [60], Creutzfeldt-Jakob disease (CJD) [63], Gerstmann-Sträussler-Scheinker (GSS) syndrome [96], and fatal familial insomnia [98,99] in men, natural scrapie in sheep, goats [86,123] and mufflons [124], transmissible mink encephalopathy in ranch-reared mink [22], chronic wasting disease of mule deer and elk [120,121], bovine spongiform encephalopathy or "mad cow disease" [14,15, 30,118] and its analogues in several exotic species of antelopes [38,55,74,79] and wild felids in zoological gardens [122], and feline spongiform encephalopathy in domestic cats [125].

This short review summarizes the history of the research to find the nature of the scrapie agent, especially as I have witnessed it unfolding before my eyes. I was fortunate enough to work with D. Carleton Gajdusek, one of the greatest scientists of the 20th century and later a personal friend and a "father-like" figure for me, and I was privileged to meet many other

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researchers who have dominated the field since the mid-1980s and some of whom are still active in the field, including Paul W. Brown, Ray Bradley, Moira Bruce, Byron Caughey, Bruce Chesbro, Heino Diringer, Alan G. Dickinson, Hugh Fraser, Peter H. Gibson, James W. Ironside, Martin Jeffrey, Michael Katz, Richard H. Kimberlin, Colin Masters, and Bob Will. I also met some others – Geoff Millson, Pat Merz, Henry Wisniewski, Shirley Lindenbaum, and, notorious albeit not always wrong, Harash Narang. In 1984, in a famous meeting at the New Battley Abbey, close to Edinburgh – organized by Alan G. Dickinson in his fruitless effort to discredit the prion theory, I met for the first time a second Nobel laureate in the field – Stanley B. Prusiner and later such giants as Charles Weissmann and Kurt Wüthrich. Many but not all of these individuals enriched me in both my personal and scientific life. I became interested in scrapie around the time Gajdusek won a Nobel Prize (1977) and now, I am one of the oldest active investigators in the field.

## Historical background

Scrapie (Fig. 1), 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", "die Zitterkrankheit" in Germany; "la maladie convulsive", "la maladie follie", "le tremblante", "la prurigo lombaire" in France; and "trzęsawka" in Poland). Scrapie was first reported in Spanish merino sheep in 1732 [70]. Following an outbreak of scrapie in Lincolnshire, British sheep farmers petitioned the Parliament to introduce some legal regulations to stop the movement of sheep. The importation of Spanish merino sheep introduced scrapie to many sheep flocks in the United Kingdom and elsewhere. One of the earliest scientific reports on scrapie had been published in the *Agricultural Improvement Society at Bath* (later changed to *Bath and West Society*) (anonymous, 1788) and, as a paragraph in the *General View of the Agriculture of Wiltshire*, published by Thomas Davies in 1811 [86]. The paper in *Agricultural Improvement Society at Bath* stated that "*within these few years* [*scrapie*] *has destroyed some in every flock around the country and made great havoc in many*" [cited after 70]. In continental Europe, scrapie was rampant in Germany and Silesia (now Poland) but disappeared from most of Central and Eastern Europe among *most of the German sheep* after 1945.

**Fig. 1.** A scrapie-affected sheep. Courtesy of Dr. Mark Dagleish, Moredun Research Institute, Edinburgh, Scotland.

In 1811, the description of scrapie, appearing in *General View of the Agriculture of Wiltshire* [cited after 86], noted:

"This disorder, we must observe, has tended, more than all other reasons combined, to bring the Wiltshire sheep into discredit. It is not clearly known when this disorder first made its appearance in Wiltshire, nor is it certain that it is peculiar to this kind of sheep. The symptoms are that the animal becomes loose in its backbone, with shakings in its hind-quarters, preceded by a continued drooping of the ears. It was very little noticed in Wiltshire till about twenty-five years ago, and yet it is certain that a disease which was undoubtedly the same disorder was known *in Lincolnshire about sixty years ago*.

By a memorial delivered to the House of Commons in 1755 by the breeders and feeders of sheep in the country of Lincoln, it is stated that for ten years then past a disorder which they called the *rickets* or *shaking* had prevailed among their sheep; that it was communicated in the blood by the rams, and would frequently be in the blood twelve months or two years before it was perceivable, but that when once a sheep had this disorder it never recovered.

The disorder called the rickets is now [1811] prevalent in some parts of Cambridgeshire, with the symptoms above mentioned.

I am informed that all sorts of sheep are subject to this disorder, though known by *various* names; and that continuing the same breed without introducing rams from other flocks (provincially – *breeding in-and-in*) will produce it. The reason, perhaps, why this complaint has been lately known as the Wiltshire disorder, is that most of the Wiltshire wethers are sold off when lambs, and are fattened before they are two years old, and the pushing them with high keep at so early an age will most assuredly discover the *goggles* if they be in the blood. [Cf. Present-day view in Roxburghshire that high feeding brings out "scrapie".]

Many thousands that have been sold, not only from Wilts but also from Hunts and Dorset, have been attacked with this disorder. The sellers have been obliged to stand by the loss, and the sort of sheep has been, in consequence, brought into discredit. It has been, however, for a long time on the

decline, and if care be taken in selecting the rams it will probably soon wear out. [italics mine. – J. P. M'G.]"

And in another paper published in 1815 in *General View of the Agriculture of Dorsetshire* [cited after 86]:

"The *goggles* have been very fatal to the sheep in this county, but it is believed this disorder is not so prevalent as was the case some years ago. Mr Balson of Athelhamptom has suffered much in his Dorset flock by this malady, and is now exchanging them for South Downs principally for that reason. The disorder is believed to be infectious or hereditary [marked by P.P.L.], and a medical gentleman attempted in vain to discover the cause. The sheep when affected with this disease rub themselves very much and reel about as if intoxicated. No cure has been discovered for this singular malady except changing the flock be deemed a remedy."

In 1848, Roche-Lubin [111] claimed that scrapie was caused by sexual overactivity of rams or, alternatively, by thunderstorms. M'Gowan [86] himself suggested the parasitic protozoan *Sarcosporidium* as the causative agent.

The first who believed that scrapie ("*tremblante*") represented a viral (caused by a "filterable agent") disease was Besnoit in 1899 [13] while the transmissible nature of scrapie was proved in late 1930s by the seminal experiments of Cuille and Chelle [32-37]. The contention that scrapie was an infectious disease caused by a filterable agent was accepted with a long-lasting scepticism. In 1938, W.S. Gordon, a deputy director of the famous Moredun Institute in Edinburgh, Scotland, repeated the experiments of Cuille and Chelle using 697 animals of which some 200 developed scrapie [64,107]. He also inadvertently proved the transmissibility of the disease using Louping ill vaccine based on formalin-fixed sheep brains [64]. World War II interrupted scrapie research which had been continued practically only by D.R. Wilson [123]. Wilson's research remained largely unpublished, as he was reluctant to present data on such an unorthodox pathogen, but the scrapie community had been and still are well aware of the unusual properties of the scrapie agent, in particular, its resistance to formalin and high temperature, and also to ionizing radiation.

The transmission of scrapie from sheep to mice by Morris and Gajdusek [105] and from goats to mice by Chandler [23-26] enabled wide-scale laboratory research and production of whimsical hypotheses, with a mean frequency approaching one every year or two. Thus, the infectious agent had been claimed to be a self-replicating membrane [7,62,71-73] or a subvirus (not well envisaged) linked to a membrane with a "linkage substance" [1,2], a viroid [50-52,88,89,92-95] and a spiroplasma [12] or a retrovirus-like element [4-6,91,114,116]. Suffice it to say that none of these hypotheses could be subsequently substantiated despite an exhaustive use of all methods of both classical and molecular virology [82].

In particular, Gordon D. Hunter, who arrived in Compton just in time to use the first mouse model developed by Chandler, worked with Millson, Kimberlin, David Haig and Michael Clark to build the basis of "scrapie biochemistry". In particular, he showed that scrapie infectivity passed through ultrafilters with the lowest limit of 30 nm and was highly hydrophobic ("sticky"). Along with Tikvah Alper at the Hammersmith Hospital they irradiated scrapie preparations to reduce scrapie infectivity and, on the basis of this, to deduce the nature of the scrapie agent. They interpreted the results according to the "target theory" by Douglas Lee which simply stated that inactivation of a given molecule reflects a "hit" to the "target" – the larger the target, the easier the hit, resulting in inactivation. In a paper published in 1977, they explicitly stated their conclusion in the title: "The scrapie agent: evidence against its dependence for replication on intrinsic nucleic acid".

## Kuru – from the obscure disease of sheep to human danger

D. Carleton Gajdusek, a young virologist and paediatrician trained by giants of the 20th century science, including Linus Pauling, Max Delbrück and MacFarlane Burnet (Fig. 2), arrived in the Territory of Papua New Guinea in March 1957, where he met Vin Zigas [81], a Lithuanian escapee from a communist regime and the Medical Officer at Kainantu in the Eastern Highland District, to investigate the cause of a strange encephalitis-like disease identified two years earlier. An interesting characterization of Gajdusek was given by Sir MacFarlane Burnet [54, p. 41]:

"I was very pleased to get your letter clarifying your attitude towards Gajdusek's rather extraordinary intrusion into New Guinea, and I thought it might be helpful if I gave you some unofficial and informal background about him.

He is quite an extraordinary individual of American birth but brought up as a child in Central Europe and multilingual. There is no question about his intelligence or training in paediatrics and virology, and I found myself very interested by his enthusiasm for the paediatric and cultural study of the development of children in primitive communities. On the other hand, his personality is quite extraordinary, and is almost legendary amongst my colleagues in the U.S. [John]

**Fig. 2.** D. Carleton Gajdusek during one of his research travel to Papua New Guinea. A gift from late D. Carleton Gajdusek.

Enders (Boston) told me that Gajdusek was very bright but you never knew when he would leave off work for a week to study Hegel or a month to go off to work with Hopi Indians. Smadel at Washington said the only way to handle him was to kick him in the tail, hard. Somebody else told me he was fine but there just wasn't anything human about him."

Gajdusek, who already had heard about this strange affliction from Roy Scragg, then a Director of Public Health, at Port Moresby, was working at that time in the laboratory of Sir MacFarlane Burnet at the Hall Institute, where he discovered autoimmune antibodies in blood samples from patients with chronic hepatitis, lupus erythematosus and multiple myeloma [57]. Burnet subsequently used these data in formulating his clonal selection hypothesis of antibody formation which won him a Nobel Prize in 1960.

In a letter to Joe Smadel, then a director at the National Institute of Health, Gajdusek described kuru [54, p. 96]:

"Emotional instability is certain; a tendency to excessive hilarity, etc. is certain. The 'mask-like' facies is rather a fixed facies – but not 'mask-like': i.e., it is full of expression but quiet, with rare blinking and little motion until stimulated. Then it responds quickly and usually somewhat excessively, with euphoric grins and smiles, or even shrieks.

The entire postural tremor situation is most complex. It is not a true cerebellar, nor a true parkinsonism tremor. It is, rather, a tremor and other types of involuntary movements which are very irregular and difficult to describe. It is definitely an antigravity postural disorder. Thus, sleeping, or when curled up and firmly supported in any position – i.e., in another person's arms, pressed tightly in the another's grasp, etc. – the tremor disappears. Any sudden relaxation of this passive support, or sudden shift of even one portion of the body's posture (as of the head, neck, one arm), produces a violent tremor plus choreiform response in the entire body, apparently aimed at re-establishing a difficult-to-maintain antigravity equilibrium. Toes are constantly gripping and searching when a patient tries to stand unaided – or, if more advanced, even when supported. Sudden loss of antigravity postural support, given passively by an examiner to head or upper extremities, suddenly sets off repetitive, irregular tremors or a choreiform pattern of movement. Rigidity is minimal, if at all present. It appears late. Instead, there is an increased tone to the muscles that are associated with attempts at maintaining posture and preventing the antigravity tremors which fight the slightest instability of standing, sitting, lying, head posture, etc. and which initiate as a startle response. If well and firmly supported passively, even in late cases, this 'intermittent rigidity' subsides to complete relaxation".

There have been never-ending discussions, even enhanced recently by the passing of Gajdusek and Gibbs, about who first said what and where, whose ideas were right and whose were wrong and who should be granted credit for the seminal discovery of the aetiology of kuru. Gajdusek once told me that all those people behaved like small children, who constantly asked "and who? and what? and who? and why?"; many of those questions are without much sense, some ideas on the aetiology of kuru became obvious from the beginning and were put to laboratory testing – specially any attempts to isolate a virus were in vain [59]. Gajdusek was fully aware that the virus hypothesis should be tested – he even delayed his departure to Papua New Guinea waiting for buffered glycerine to preserve a putative "kuru virus" before attempts to isolate it on the chorioallantoic membranes of chick embryos at the Hall Institute [54]. There have been also animated discussions about who was the first to point at cannibalism as a vehicle of kuru spread [85]. "Even a complete drunk would come to the conclusion that a disease endemic among cannibals must spread by eating corpses" Gajdusek told me in one of several endless discussions. However, there were other ethnic groups like the Ku Ku Ku Ku (Anga) who were also cannibals but did not developed kuru; thus, cannibalism *per se* was not enough and the necessary factor was an infectious agent, which had not been discovered at the beginning.

The situation changed dramatically when William Hadlow went to an exhibition organized by the Wellcome Trust in London where he saw photographs of kuru pathology produced by Igor Klatzo, a Polish-born neuropathologist working at the NIH. Hadlow recognized those micrographs as reminiscent of scrapie and

sent a letter to Gajdusek, who was in the bushes of Papua New Guinea at that time. "Bill Hadlow's suggestion of an analogy between kuru and scrapie came as a surprise to me and to all of us in kuru work", recalled Gajdusek in the introduction to "Kuru. Early Letters and Field Notes from the collection of D. Carleton Gajdusek" [54]. This prompted a visit of Gajdusek to the Moredun Institute in Edinburgh and to Compton, centres of scrapie research, from which Gajdusek came back with a strong conviction to inoculate chimpanzees; that task was supervised by Dr. Clarence Joseph Gibbs, Jr., who was already hired for this purpose. In 1963, Gajdusek, Gibbs and Alpers organized a meeting of "Slow, Latent, and Temperate Virus Infections", the proceedings of which were published in a book in 1965 [61]. A passage on the transmission of kuru to chimpanzee was incorporated in the text.

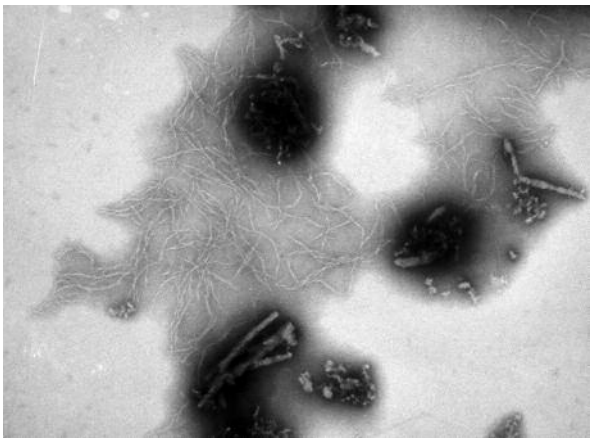

**Fig. 3.** Scrapie-associated fibrils.

## Prion hypothesis

The "prion" hypothesis, which is deeply rooted in the association between prion protein (PrP) and infectivity, was formulated by Stanley B. Prusiner in 1982 [108]. The hypothesis postulated that the scrapie agent was a proteinaceous infectious particle (actually it should read "proin" but Prusiner thought, quite rightly, that "prion" sounded better than "proin"), because infectivity was dependent on protein but resistant to methods known to inactivate nucleic acids. The idea was not completely novel; it had been meandering around for a long while. Indeed, a similar proposal was presented by Griffith [65], Levine ("Scrapie: an infective polypeptide?") [80] and Gibbons and Hunter [62], who developed the earlier suggestion of Alper *et al.* [8] that the scrapie agent was devoid of disease-specific nucleic acid. In particular, Griffith, a mathematician, proposed three hypotheses about how the protein may induce its own replication and not "cause the whole theoretical structure of molecular biology to come tumbling down". Of note, he wrote, "There is an obvious analogy between the idea presented here and the idea that a gas can only condense on nuclei which are already present". This sentence introduced a caveat of nucleation polymerization as a basis for formation of amyloids ("prionoids" by Adriano Aguzzi – [3]) which is very popular among researchers, who are climbing onto the bandwagon of prions, as an explanation for other neurodegenerative disorders.

It is very perplexing for me and I have never found a likely explanation, while it happened that several investigators who had found previously that scrapie infectivity was sensitive to proteolytic digestion [28,103], did not discover PrP.

The first to see $PrP^{Sc}$ were Patricia Merz and Henryk M. Wiśniewski, of the New York State Research Institute for Basic Research in Developmental Disabilities, who visualized abnormal fibrillar structures in scrapie- [100,101], CJD- and kuru-infected brains [9,100] by negative-stain electron microscopy (Fig. 3). It is worth stressing that her discovery was probably the last of such a caliber achieved on the basis of this beautiful but largely pushed to a shadow technique of electron microscopy. She labelled the fibrillar structures as scrapie-associated fibrils and believed they may represent the scrapie agent being a form of "filamentous viruses". The same structures were seen by Prusiner who labelled them "prion rods" and with an insight classified them as a form of amyloid. The protein itself, PrP, was first discovered by Cho [28] and Prusiner's group [97] and the association between it, infectivity and fibrils was reported almost simultaneously by Diringer [53] and Prusiner [110]. The N-terminal sequence of PrP was obtained [109] and the gene encoding PrP was cloned by Charles Weissmann, one of the greatest molecular biologists of recent times [11]. Then it was shown that the ablation of this gene led to resistance to scrapie [20,21] and the protein was indispensable for scrapie-induced neurotoxicity [16]. These seminal discoveries set a stage for the prion field to expand enormously in the last 20 years but I doubt that all of questions asked in the 1960s and 1970s have been answered.

## Is there a cofactor necessary for PrP to become an infectious agent – the virino and "unified" hypothesis

From the early work of Dickinson *et al*. [44-48], who was using methods of classical genetics, it was known that scrapie incubation period was tightly linked to the gene designated Sinc (in mice; from scrapie incubation) and SIP (in sheep; from shorter incubation period) and it was even proposed by Parry [106] that scrapie is a bona fide genetic disorder while its transmissibility is only an epiphenomenon. The discovery of Sinc was instrumental in supporting the notion that the scrapie agent has an independent genome [18]. Nowadays we know that Sinc and Sip are genes encoding PrP [19,27,104]. Different strains of the scrapie agent can be identified in terms of their stable biological characteristics, including their thermostability [115]. 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 are consistent with the effects of mutations in the genome of the agent which is presumably an as yet undiscovered disease-specific nucleic acid.

Approximately 20 strains of scrapie agent have been isolated from sheep and goats affected with clinical scrapie [19]. 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 [41]. Some sources of sheep scrapie are not transmissible to mice, for example the CH 1641 isolate [56]; but those that are, can be divided into two groups on the basis of their properties in the two homozygous *Sinc* (*Prn-i*) genotypes of mice. The ME7 group of agents exhibit a short incubation period when passaged through $Sinc^{s}$ ($Prn\text{-}p^{A}$) 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 exhibit exactly the opposite characteristics: short incubation period in $Sinc^{p7}$ mice and long incubation period in $Sinc^{s7}$ mice. It has been conclusively demonstrated that the *Sinc* gene is congruent with the *Prn-i* gene; in other words, PrP is the product of *Sinc* [19].

Passage through a species different from that used for the primary isolation (i.e., across the *species barrier*) is a useful method to separate mixtures of strains and to isolate (select) new mutant strains [31,78]. One of the best known examples 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 [77]. Two additional sets of experiments may be classically interpreted in a sense that scrapie agent must have an independent genome, for which the *orthodox* candidate is obviously, a nucleic acid.

First, strains of scrapie agent undergo changes of certain characteristics such as incubation period, lesion profile, and presence and amount of PrP amyloid deposits, which are compatible with mutations of "conventional" pathogens [17]. Three classes of strain stability have been established [17]. Class I stability strains (ME7, 22C) possess stable characteristics irrespective of the *Sinc* (s7 or p7) (*Prn-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 [17], 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.

"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*" [17]. 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 production 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. In summary, these data show the selection of mutant strains of scrapie agent in the same host species (*the same sequence variant of Prn-p gene*), indicating that the genome of scrapie agent is host-*independent*. It is worth mentioning that different strains present different thermostability but influence of the host (Sinc genotype) in those experiments was small [115].

The most compelling evidence for not fully proteinaceous nature of the infectious agent is a set of

experiments demonstrating a competition between strains. Different strains of scrapie agent can exhibit competition when inoculated at different times, either intracerebrally [43] or peripherally [42]. For example, when VM mice ($Sinc^{p7}$; $Prn\text{-}p^{B}$) were inoculated intracerebrally 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 nine weeks, the incubation period of the 22A increased by 30 days because of the competition with the slow strain inoculated first. In another experiment, R III mice ($Sinc^{s7}$; $Prn\text{-}p^{a}$), 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 which died after the expected incubation period of 22A. Furthermore, Kimberlin and Walker [78] studied blocking quantitatively and showed 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* (PrP itself!) [48,49].

The very presence of strains may be readily explained by the existence of strain-specific oligonucleotide or a ubiquitous virus; however, these objects have never been found despite several attempts to detect disease-specific nucleic acids [5,76,102]. Thus, the alternative explanation, in agreement with protein-only hypothesis, came to a turning point.

The virino hypothesis, first formulated by Dickinson and Outram [49], was based on results of classical genetic experiments and suggested that "the overdominance effect" ("overdominance" means that certain characteristics, i.e. incubation period in heterozygotes is beyond the range of it found in either of the homozygotes) indicates that the two alleles (of *Sinc*) do not act independently of one another and this gives a clue to the type of structural *Sinc* gene product on which agent replication depends. "It is suggested by analogy with the "hybrid enzymes" in yeast that the replication site determined by *Sinc* is a multimeric structure and that (the alleles of *Sinc*) contribute different sub-units of this so that it is a heteromeric structure in the heterozygote" [67]. The results of competition experiments again suggested that the informational molecule encoding the strain properties is tightly linked with the host protein (PrP). The best candidate for this "informational" molecule is a nucleic acid which has been never found, however.

In a deep insight, Dickinson and Outram [48] wrote:

"that the infectious agent is produced by the host providing coat proteins to protect the agent's independent genome, which can be very small because it needs not code for a protein product. Whatever the nature of the agent's informational molecule, it presumably acts by 'disregulating' or blocking some important step in cell metabolism. This concept of the infectious agent as an informational hybrid between host-coded molecules and the genome of the agent *per se* has been designated 'the virino hypothesis."

Charles Weissmann formulated a "unified theory" of prion propagation [117] to rejuvenate "virino" hypothesis in a more sophisticated and updated form. Weissmann proposed that the scrapie agent (a "prion" by another name or "holoprion" by Weissmann) is composed of a host-encoded protein (PrP, "apoprion") and an informational molecule probably RNA ("a coprion"). "The theory accepts the premises of a virino hypothesis that a nucleic acid responsible for some phenotypic features and is replicated in the cell, but it denies that the nucleic acid is required for infectivity and that it represents an independent agent-specific genome". Simoneau *et al.* [113] reported that recombinant PrP (recPrP) is able to transmit scrapie following a conversion to a β-sheeted form if scrapie-derived RNA is present as a cofactor. The RNA species involved consisted of approximately 27 and 55 nucleotides and was RNAse A – sensitive suggesting that they are single-stranded moieties and thus somehow related to recently discovered realm of cellular ribo-regulators – miRNA, siRNA or piRNA already evoked by Weissmann as possible candidates for co-prions [117]. Indeed Weissmann wrote "The discovery of siRNAs and microRNAs, which would have escaped notice in earlier analyses of prion preparations, owing to both their size and their host origin, provides candidates for the hypothetical co-prion that has been proposed by the Unified theory" [117]. This report opens a hot discussion in which both Diringer and Dickinson, silent for a long time, took part. The discussion points out that PrP possesses nucleic acid chaperoning properties and assists nucleic acid folding like so called "nucleic acid chaperons" [66]. In particular, the N-terminal PrP peptide HuPrP(23-145) but not HuPrP(122-231) facilitates DNA strand exchange to obtain perfectly matched dsDNA; enhances the rate of

ribozyme-mediated cleavage and finally, PrP enhances RNA trans-splicing. This activity of PrP was analogous to that of HIV-1 NCp7 protein. Furthermore, using serial protein misfolding cyclic amplification, Supattapone and colleagues [39] found that conversion of $PrP^{c}$ to $PrP^{Sc}$ requires co-factors, namely mammalian but not invertebrate ssRNA but not dsRNA. The latter finding is in contrast to data posted by Karapetyan [75] who, using antibodies against dsRNA, demonstrated dsRNA overexpressed in scrapie-infected cells *in vitro* and brains. Furthermore, in a later publication [40], the Supattapone's group reported that RNA amplified conversion in sPMCA format of $PrP^{c}$ to $PrP^{Sc}$ in hamsters but not mice or voles. Lastly, Saba *et al*. [112] found deregulation of certain class of miRNA in experimental scrapie.

Collectively, the potential "coprion" RNA has yet to be found. But the numerous data point out that an additional cofactor, probably a short stretch of RNA, may be a "missing link" in searching for the scrapie agent.

## Transmissible spongiform encephalopathies versus PrP proteinopathies

Not all prion diseases have been transmitted and perhaps not all are transmissible. Those which are not transmissible are *PrP* proteinopathies, diseases of accumulation of misfolded *PrP*. There is a caveat of modeling those diseases using transgenic mice technology. For instance, it was reported that transgenic mice expressing a *PrP* gene mutation at codon 101 (Tg(GSS MoPrP), which is analogous to codon 102 mutation associated with GSS in man [68,69] develops "spontaneous degeneration". Interestingly, Tg(GSS MoPrP) mice were originally reported to be devoid of $PrP^{sc}$ on Western blot [69]. Furthermore, the report of the transmission of scrapie-like disease from the brains of Tg(GSS MoPrP) mice [69] was followed by a report of the analogous transmission from the brains of transgenic mice constructed with non-mutated (normal or wild) hamster and sheep *PrP* gene. In these wtTg(Ha PrP) mice, the spontaneous neurodegenerative disorder in the form of necrotizing myelopathy and demyelinating polyneuropathy developed after a prolonged time [119]. These mice do not produce $PrP^{sc}$ but they apparently transmit disease to Syrian hamsters, analogously to Tg(GSS PrP) mice. The latter finding may suggest that the number of the *PrP* transgenes and not merely presence of mutation at codon 101 is responsible for the development of neurodegeneration. Indeed, when analogous mice were constructed by means of reciprocal recombination (thus, without extra copies of the transgene) [10,90], neither "spontaneous neurodegeneration" nor "transmission of the disease" have been observed. However, these Tg mice were very susceptible to infection with the GSS inoculum, a disease otherwise difficult to transmit.

Thus, these transgenic mice may represent true "*prion protein disorders*", i.e., PrP proteinopathy in which alterations (amplification) of the *PrP* gene cause "spontaneous neurodegeneration". However, the absence of transmission from Tg mice without overexpression of the transgene clearly suggest that overexpression itself and not the "genetic construction" of prion is responsible for the "spontaneous neurodegeneration". In conclusion, the Hsiao *et al*. [69] experiment once regarded as the most convincing to support the prion theory seems much less convincing in the light shed by reciprocal recombination expe-riments. It is also true, however, that the nature of transmission from brains of Tg(GSS MoPrP) remains an enigma.

This first report of "spontaneous" neurodegeneration in Tg mice overexpressing PrP was followed by many others. Before discussing one of many examples, a short digression about the definition of the word "infectious" may be instructive. According to the 28th edition of Stedman's Medical Dictionary, the term infectious "denotes a disease due to the action of a microorganism". Thus, in my mind at least, it is entirely wrong to describe synthetic PrP peptide as "infectious" even if those structures replicate. Paul Brown, in his lecture at the Neuroprion meeting in Montreal in 2011, said that you may think of rust as a parallel; rust on a metal surface expands (i.e., "replicates") but nobody would call it "infectious".

The data on "transmission" of amyloid fibrils to transgenic animals that harbour a high copy number of a transgene that encodes the protein the fibrils are composed of, seem only to reflect a process of seeding, a nucleation of amyloid which Gajdusek envisaged almost 15 years ago [58]. A transmission to wild type animals is more important and more "natural" [29]. To this end, Makarava *et al*. [87] showed that "prion infectivity" (i.e., nucleation) may be achieved in hamsters inoculated with rPrP of cross-β-sheeted amyloid structures. The disease was clearly different from TSEs in hamsters both by the size of the $PrP^{res}$ peptide (16 kDa in contrast to 27-30 kDa following proteinase K treatment), unusually slow disease progression (hence the name of the strain

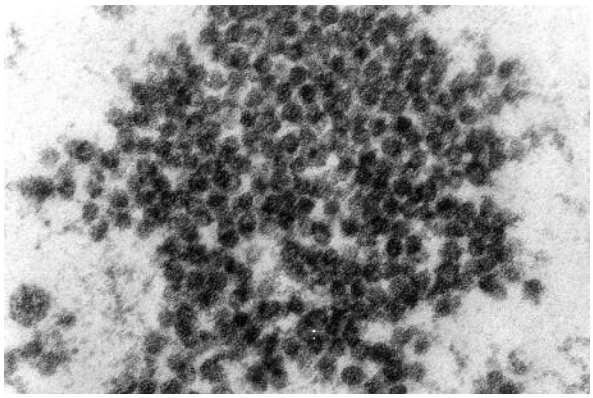

**Fig. 4.** Tubulovesicular structures (TVS) in a case of vCJD kindly supplied by Prof. James W. Ironside, Edinburgh, Scotland.

"SSLOW") and different pattern of PrP immunostaining. What is even more surprising, the recPrP did not initiate a disease after the first passage, but did it in the second passage. This phenomenon may be explained by different structures of rPrP and $HaPrP^{Sc}$, which is in agreement with a hypothesis of seeding as a mechanism underlying PrP proteinopathy. In a second paper, these authors introduced the hypothesis of "deformed templating" to explain seeding of cross-β-sheeted structures by a nucleus substantially different from the seeded protein [87]. Also, according to the "unified theory" of Weissmann [117], the rPrP alone may lack an important cofactor, probably RNA.

In a second paper, brain homogenate containing "atypical" PK-resistant rPrP of 13-21 kDa (as opposed to typical $HaPrP^{Sc}$) was inoculated into Syrian hamsters but no disease was observed despite the large amount of both typical and atypical $PrP^{Sc}$; the authors labelled the new "strain" LOTSS (i.e. Low Toxicity Synthetic Strain) and showed that it is conformationally different from SSLOW and 263K [87]. As in previous studies, conversion of LOTSS rPRP into $PrP^{Sc}$ was enhanced in the presence of RNA.

Here we encounter a new and the most important caveat – how to discriminate between true TSEs and amyloid prion proteinopathies. In my mind, electron microscopy should be used to detect, in coded samples, so-called "tubulovesicular structures" (TVS) (Fig. 4). These structures are virus-like particles, measuring 25-30 nm in diameter and disease specific for all TSEs [83,84]. If in PrP proteinopathies, TVS are found – it means that these diseases realistically model true "prion" disease; if not, they are merely proteinopathies or transmissible brain amyloidoses.

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