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Amyloids, prions and the inherent infectious nature of misfolded protein aggregates

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Misfolded aggregates present in amyloid fibrils are associated with various diseases known as 'protein misfolding' disorders. Among them, prion diseases are unique in that the pathology can be transmitted by an infectious process involving an unprecedented agent known as a 'prion'. Prions are infectious proteins that can transmit biological information by propagating protein misfolding and aggregation. The molecular mechanism of prion conversion has a striking resemblance to the process of amyloid formation, suggesting that misfolded aggregates have an inherent ability to be transmissible. Intriguing recent data suggest that other protein misfolding disorders might also be transmitted by a prion-like infectious process.

Protein misfolding disorders

The biological function of cells depends on the correct folding of a network of thousands of proteins. The information required to fold a protein into a functional, specific three-dimensional structure is contained in its amino acid sequence. In general, proteins fold properly into their native conformation and, if they do not, the misfolding is corrected by chaperone proteins [1]. In protein misfolding disorders (PMDs), however, misfolding of a protein results in its aggregation and accumulation as protein deposits in diverse tissues [2–5].

Among the PMDs are Alzheimer's disease, Parkinson's disease, Huntington's disease, transmissible spongiform encephalopathies (TSEs), serpin-deficiency disorders, hemolytic anemia, cystic fibrosis, diabetes type II, amyotrophic lateral sclerosis, secondary amyloidosis, dialysis-related amyloidosis and more than ten other rare diseases [2–5]. Although the proteins involved in these diseases do not share sequence or structural identity, all of them can adopt at least two different conformations without requiring changes in their amino acid sequence. The misfolded form of the protein usually contains stacks of β sheets organized in a polymeric arrangement known as a 'cross-β' structure [6]. Because β sheets can be stabilized by intermolecular interactions, misfolded proteins have a high tendency to form oligomers and larger polymers.

Compelling data from biochemical, genetic and neuropathological studies support the involvement of protein misfolding and aggregation in the pathology of PMDs [5]. For example, abnormal aggregates are usually present in the tissues with most damage [7,8], and accumulation of these deposits in diverse organs is the endpoint in most PMDs [2,5]. Mutations in the gene encoding the misfolded protein produce inherited forms of the disease, which usually have an earlier onset and a more severe phenotype than the sporadic forms [9]. Transgenic animals expressing a human mutant gene for the misfolded protein develop some of the neuropathological and clinical characteristics typical of the human disease [10]. Finally, misfolded protein aggregates produced *in vitro* are toxic to cells and induce apoptosis [11].

Here, we review recent findings indicating that other PMDs might be transmitted through a mechanism involving replication of protein misfolding and aggregation.

The intriguing mechanism of prion diseases: an infectious protein

The crucial role of the protein misfolding process is perhaps most clear in the prion disorders [12], which are also called TSEs and are the only member of the PMD group known to be transmissible by infection. TSEs comprise a group of infectious neurodegenerative diseases that affect humans and other animals, and are characterized by brain vacuolation, astrogliosis, neuronal apoptosis and accumulation of the misfolded, protease-resistant prion protein (termed PrP^{Sc}) in the central nervous system [13,14]. Despite being rare diseases, TSEs have gained considerable notoriety through emergence of the new variant form of Creutzfeldt–Jakob disease, which is most probably caused by the consumption of meat infected with bovine spongiform encephalopathy [15].

The nature of the prion infectious agent and its mechanism of propagation represent one of the most debated and intriguing issues in recent biology [16]. Compelling evidence strongly supports the concept that the misfolded prion protein (PrP^{Sc}) is the only component

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Box 1. Evidence supporting the prion hypothesis

Compelling evidence has accumulated over the years to support the protein-only hypothesis of prion infectivity:

- The infectious agent is not affected by procedures that normally destroy nucleic acids [57].
- The infectious material is too small to be a conventional type of microorganism [32,58].
- Despite much effort, no virus or nucleic acid has been consistently associated with the agent [59].
- Highly purified preparations of PrP^{Sc} transmit the disease [60].
- Infectivity is proportional to the PrP^{Sc} concentration [61].
- Procedures that inactivate PrP^{Sc} markedly reduce or eliminate infectivity [62].
- PrP^{C} knockout mice are not susceptible to infection [63].
- All inherited cases of TSE are linked to mutations in the PrP gene [13].
- Transgenic mice overexpressing the PrP gene spontaneously develop neuropathological and clinical features of TSEs [64].
- PrP^{Sc} can induce the conversion of PrP^{C} *in vitro* in an autocatalytic fashion [22,65].

of the infectious agent and that it can 'replicate' in the brain in the absence of nucleic acid by converting the natively folded prion protein (PrP^{C}) into a misfolded form [13,16] (Box 1). Nevertheless, the widely accepted 'final proof' – namely, the generation of infectious prions by inducing misfolding of the prion protein *in vitro* – has remained elusive [16]. Two recent studies have, however, provided significant steps forward in this direction [17,18].

Legname *et al.* [17] showed that a recombinant fragment of mouse PrP (comprising residues 89–230) assembled into amyloid fibrils induced a TSE-like disease with PrP^{Sc} formation when injected into transgenic mice overexpressing the same PrP sequence. The disease was subsequently transmitted to wild-type mice in a second passage. Although this study provides interesting data, there are several limitations in the experimental design. First, infectivity was originally observed only in mice that were highly overexpressing (16-fold higher than in wild type) a truncated version of PrP, which is a matter of concern because this type of mouse can spontaneously develop a prion-like disease [19–21]. Second, a crucial missing control was the inoculation of mice with a brain homogenate of transgenic mice that had not been injected with synthetic prions; this control is important because the same group previously reported examples of the spontaneous generation of infectivity in transgenic mice overexpressing other mutant PrP molecules. Last, the infectivity per unit of PrP was at least a billion-fold lower than it should be, given that the fibril-inoculated transgenic mice became ill after very long incubation times.

We and our co-workers [18] recently reported the generation of infectivity *in vitro* by cyclic amplification of the prion replication process (Figure 1). For these studies we used the 'protein misfolding cyclic amplification' (PMCA) – technology that we devised to mimic, in an accelerated manner, the process of prion propagation that occurs during the development of disease in the brains of affected individuals [22]. Via PMCA, we converted large quantities of PrP^{C} using minute amounts of a brain-derived PrP^{Sc} template. By carrying out serial dilutions,

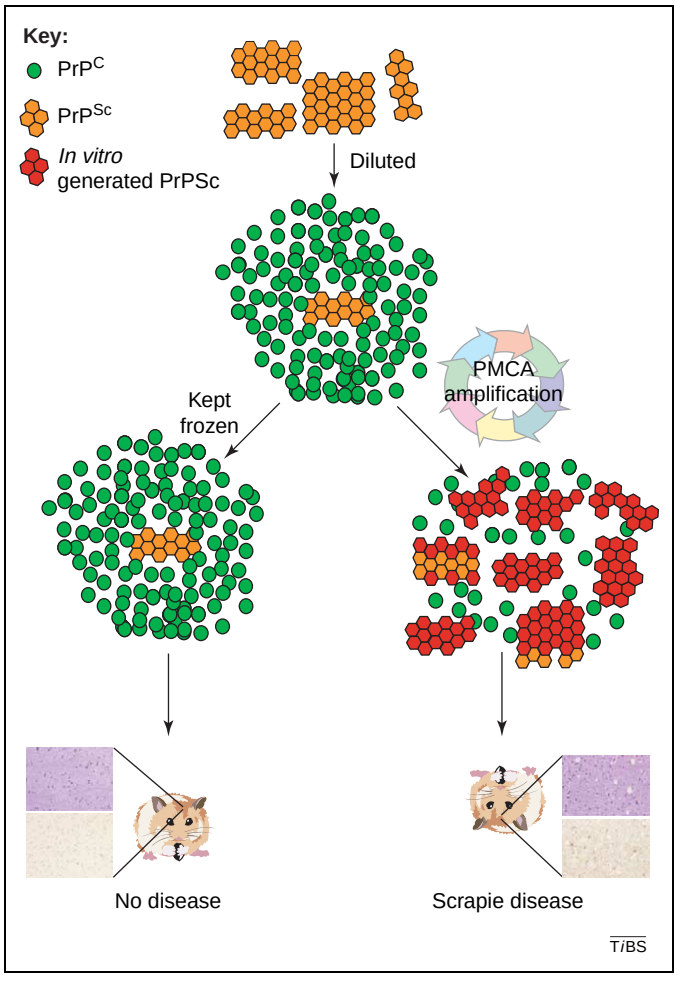

Figure 1. *In vitro* generation of infectious prions. Subjecting a solution of highly diluted brain-derived PrP^{Sc} in an excess of PrP^{C} to many cycles of protein misfolding cyclic amplification (PMCA) resulted in amplification of the amount of PrP^{Sc} at the expense of the normal protein [18]. When the *in vitro* generated PrP^{Sc} was inoculated into wild-type hamsters, all of the hamsters developed a disease with clinical, histological and biochemical characteristics typical of scrapie. Control hamsters inoculated with the original diluted material without amplification remained free of the disease.

the prions could be maintained indefinitely in a replicating state at the expense of PrP^{C}, largely after all of the original PrP^{Sc} protein molecules had been eliminated [18]. When PrP^{Sc} produced *in vitro* by this method was inoculated into wild-type hamsters, all of the hamsters succumbed to the disease at ~170 days after inoculation (Figure 1). Importantly, the disease showed the clinical, histological and biochemical characteristics typical of scrapie and was subsequently transmitted to other hamsters [18]. Because the whole-brain homogenate from healthy hamsters was used as a source of the PrP^{C} substrate, however, we cannot rule out the possibility that other molecules might be involved in prion infectivity.

Prion replication is thought to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^{C}, catalyzing its conversion to the pathogenic form of the protein. The precise molecular mechanism of PrP^{C} to PrP^{Sc} conversion is not well understood; however, the data available support a model in which infectious PrP^{Sc} is an oligomer that acts as a seed to bind PrP^{C} and to catalyze its conversion into the misfolded form by incorporation into the growing polymer [23,24] (Figure 2a).

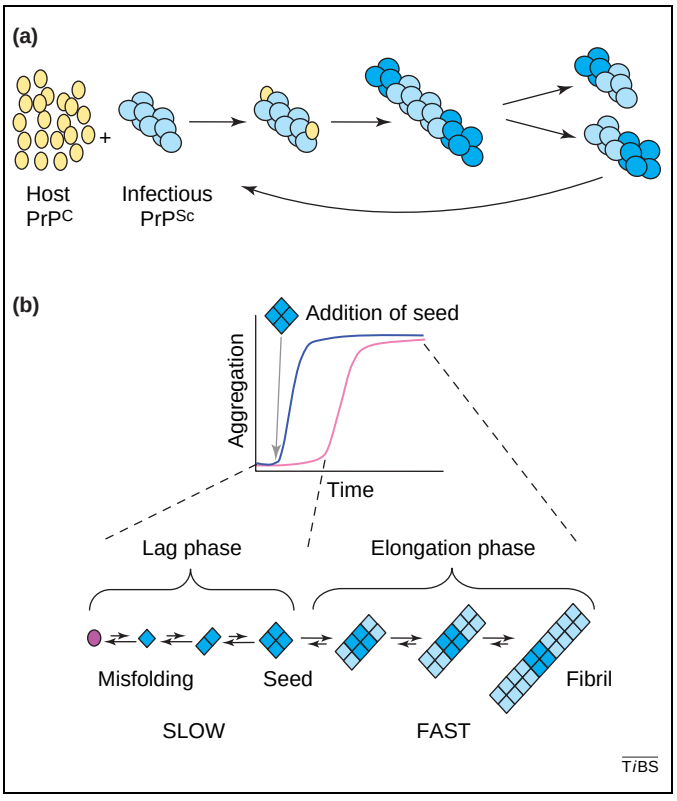

Figure 2. Mechanism of prion replication and the seeding–nucleation model of amyloid formation. Compelling evidence suggests that both PrP^{C} to PrP^{Sc} conversion **(a)** and amyloid fibril formation **(b)** follow a crystallization-like process known as 'seeding–nucleation'. In this model, formation of a stable oligomeric structure that is capable of further sustaining and catalyzing polymerization of the protein is the key and kinetically limiting step. (a) In prion diseases, PrP^{C} is rarely able to convert spontaneously into the oligomeric PrP^{Sc}, but exogenous addition of the misfolded protein acts as a seed to induce exponential conversion of the native PrP^{C} protein. (b) Amyloid formation consists of two kinetic phases. In the 'lag phase', oligomeric nuclei are formed in a slow process that involves misfolding of the protein and unfavorable intermolecular interactions. Once these 'seeds' are formed, a much more rapid 'elongation phase' results in fibril formation. The limiting step in the process is the formation of seeds to direct further aggregation [26]. Amyloid formation can be substantially accelerated by the addition of preformed seeds (blue line) representing the structure that is inherently infectious. Part (b) modified, with permission, from Ref. [24].

At some point, the long PrP^{Sc} polymers break into smaller pieces either by a mechanical force or by catalysis in an unknown process. This fragmentation enables the increased numbers of effective nuclei to direct further conversion of PrP^{C}.

The view that the transmission of biological information by the propagation of protein misfolding is the exclusive domain of pathologies associated with a rogue prion protein has changed markedly with the discovery that some proteins behave as prions in yeast and other fungi (Box 2).

The infectious nature of misfolded aggregates

Similar to the way in which PrP^{C} is converted into PrP^{Sc} in TSE, the protein conformational changes associated with the pathogenesis of most PMDs result in the formation of abnormal proteins that are rich in β-sheet structure, are partially resistant to proteolysis, and have a high tendency to form larger-order aggregates [2–5]. Indeed, a common feature of several PMDs, including TSE, is the aggregation and deposition of the misfolded protein in different organs in the form of amyloid-like plaques.

Box 2. The yeast prions

In a visionary article, Reed Wickner [66] expanded the prion concept to explain the unusual non-mendelian transmission of two yeast genetic elements termed [URE3] and [PSI^{+}], which he proposed were the prion forms of the Ure2 and Sup35 proteins (Ure2p and Sup35p), respectively. A yeast prion has been defined as an infectious protein that behaves as a non-mendelian genetic element and transmits biological information in the absence of nucleic acid [67]. Although yeast prions have been discovered relatively recently, the remarkable progress in this area has provided some important implications for understanding the protein-only nature of the 'infectious agent'.

The first breakthrough came from studies by Sparrer *et al.* [68], which showed that introducing *in-vitro*-converted purified Sup35 prion domain (N-terminal residues 1–254) into yeast cells led to the appearance of [PSI^{+}] prion in 1–2% of transformed cells. Maddelein *et al.* [69] subsequently showed that inserting fibrils made *in vitro* from renatured recombinant HET-s into the mycelia of *Podospora anserina* caused the efficient appearance of [HET-s] prions [69]. A recent study by King and Diaz-Avalos [70] has shown that, when transformed into amyloid fibrils by incubation with yeast-derived infectious aggregates, bacterially produced N-terminal fragments of Sup35p labeled with green fluorescent protein propagated the prion phenotype to yeast cells [70]. Moreover, the *in-vitro*-converted protein faithfully propagated the characteristics of several strains used to begin the conversion reaction. The same result has been obtained independently by Weissman and co-workers [71]. They found that amyloid fibrils produced *de novo* from the recombinant Sup35 prion domain at different temperatures adopted different, stably propagating conformations. Infection of yeast with these different amyloid conformations led to distinct [PSI^{+}] strains *in vivo* [71].

Interestingly, the data available indicate that amyloid formation in all disorders follows a seeding–nucleation mechanism [25]. Analogous to a crystallization process, amyloid formation depends on the slow interaction of misfolded protein monomers to form oligomeric nuclei, around which a faster phase of elongation takes place (Figure 2b). Similar to PrP^{Sc} in TSEs, the oligomeric nuclei act as a seed to induce and to stabilize conversion of the native monomeric protein. The limiting step in this process is nuclei formation, and the extent of amyloidosis depends on the number of seeds produced [25,26]. These theoretical considerations are supported by extensive experimental data, and the view that protein misfolding and aggregation follow a seeding–nucleation mechanism is widely accepted [27–29].

The seeding–nucleation model provides a rationale and plausible explanation for the infectious nature of prions and suggests that protein misfolding processes, such as those associated with several human diseases, have the inherent ability to be transmissible. Infectivity lies in the capacity of preformed stable misfolded oligomeric proteins to act as a seed to catalyze the misfolding and aggregation process [30] (Figure 2b). The acceleration of protein aggregation by the addition of seeds has been convincingly reported *in vitro* for several proteins implicated in diverse PMDs [27–29]. Extrapolating the *in vitro* results to the *in vivo* situation suggests that the correct administration of a pre-aggregated, stable, misfolded structure should substantially accelerate the misfolding, aggregation and tissue accumulation of the protein. Provided that protein misfolding and aggregation are the cause of the disease, this seeding should lead to the acceleration of a pathogenic

process that, in the absence of the seed, was set to occur much later in life or not at all during the lifespan of the individual.

Are other protein misfolding disorders infectious?

The transmissibility of amyloidosis and other PMDs has not been thoroughly investigated [31], but it is generally assumed, on the basis of epidemiological studies, that these disorders do not have an infectious origin. For example, family members or medical professionals working with individuals with PMDs do not have a higher propensity to develop the disease. The same is true, however, for prion diseases. It should be emphasized that the principles that generally apply to conventional infectious diseases do not necessarily hold true for this protein-only agent, which follows a complicated mechanism of transmission and requires special routes of infection. In addition, the putative long incubation times (up to several decades in humans) further hinder tracking of the potentially infectious origin. The challenges involved in clearly establishing an infectious cause will probably be even greater in more prevalent diseases, such as Alzheimer's or diabetes type 2.

Several reasons can explain the failure of some amyloid aggregates to act like prions, even though they have the ability to do so. First, there might be a problem of bioavailability if the aggregates are unable to reach the correct tissue and/or the right subcellular compartment to propagate the misfolding. This is especially likely to be a problem for some of the intracellular aggregates such as Parkinson's Lewy bodies or Huntington's intranuclear aggregates. Second, some of the oligomeric 'infectious' seeds might be unstable and unable to resist biological clearance. The high resistance of PrP^{Sc} to proteases and extreme conditions might be key to the efficiency of this protein as an infectious agent [12]. Last, the formation of hyperstable and large aggregates is likely to be a hindrance in propagating the misfolding and aggregation [32]. Indeed, from our findings with the PMCA amplification of mammalian prions [22] and from studies of the replication of yeast prions [33], it seems clear that fragmentation of aggregates is essential for effective propagation.

Perhaps the best way to investigate the infectious propagation of PMDs is by attempting to transmit the disease to experimental animals. Herein lies a crucial difference between TSEs and other PMDs: whereas TSEs naturally affect humans and other animals [14], most PMDs are restricted to humans. In addition, the experimental transgenic animal models for PMDs recapitulate only partially the characteristics of the human disease [10], whereas the animal models of TSEs perfectly reproduce all features of human prion disorders [34]. Despite this fact, human prions cannot infect wild-type rodents because of the species barrier phenomenon [35]. Even in transgenic mice expressing the human gene, transmissibility from humans is not efficient unless the mouse gene is removed [35]. By extrapolating these findings to other PMDs, it is obvious that selecting the right experimental model is crucial to maximize the probability of successful transmission.

Evidence for the transmission of other PMDs

Alzheimer's disease

Several attempts have been made to transmit Alzheimer's disease to experimental animals with intriguing, but conflicting, results [36–40]. Marmosets injected with brain homogenates from individuals with Alzheimer's disease developed scattered deposits of the amyloid-β protein (Aβ) in the brain parenchyma and cerebral vasculature 6–7 years after inoculation [41]. Interestingly, the resultant amyloid lesions were not limited to the injection site, but had spread well beyond into the brain. Nevertheless, it is not possible to exclude the possibility that these deposits came from the material injected. In addition, extensive studies by Gajdusek and colleagues [39] failed to transmit Alzheimer's disease and other dementias to primates. Because in most of these studies a single primate was used for inoculation, it will be important to repeat some of these experiments with a larger cohort of animals.

More recent studies have used transgenic mice expressing the human mutant amyloid precursor protein gene. Walker and co-workers [42,43] infused diluted brain homogenates, derived from individuals with Alzheimer's disease, unilaterally into the hippocampus and neocortex of 3-month-old transgenic mice. Up to 4 weeks after infusion there was no deposition of Aβ in the brain; after 5 months, however, transgenic mice developed profuse Aβ-immunoreactive senile plaques and vascular deposits exclusively in the hemisphere injected [42]. After 12 months, abundant deposits of Aβ were present bilaterally in the forebrain, but plaque load was still clearly greater in the injected hemisphere [43]. These findings clearly show that preformed Aβ aggregates can enhance the deposition of plaques *in vivo*. Because these transgenic mice 'spontaneously' develop Alzheimer's disease pathology later in life, it is not possible to conclude whether the Alzheimer's disease brain acted as an infectious agent or as an accelerator of a process that was genetically programmed to occur.

Reactive systemic amyloidosis

Perhaps the best evidence for a prion-like phenomenon in other PMDs comes from the pioneer studies of the groups of Westermark and Higuchi, who are working on systemic amyloidosis associated with the deposition of Amyloid-A (AA) and apolipoprotein AII (apoAII) amyloid, respectively.

AA is a 76-residue N-terminal cleavage product of serum amyloid-A (SAA) protein, an acute-phase reactant apolipoprotein [44,45]. The plasma concentration of SAA is normally low at ~20 mg/l, but it can increase to >1,000 mg/l as a result of an inflammatory stimulus [46]. Under these conditions, AA is deposited systemically as amyloid in vital organs including the liver, spleen and kidneys [45]. Clinically, AA amyloidosis occurs in individuals with rheumatoid arthritis and other chronic inflammatory diseases [44]. The disease can be induced experimentally in mice by an inflammatory stimulus that markedly increases the concentration of SAA [47]. After 2–3 weeks, the mice develop systemic AA deposits similar to those found in individuals with AA amyloidosis.

This lag phase is markedly shortened to few days when mice are given, concomitantly, an intravenous injection of an extract from the spleen or liver containing amyloid plaques [48]. This tissue preparation is often referred as 'amyloid enhancing factor' (AEF) [49]. Although the nature of AEF has been extensively debated, recent experiments have shown that the active principle in the AEF is the amyloid fibril itself [50,51].

Interestingly, it has been shown that inoculating animals with AEF even at minuscule doses leads to acceleration of the disease and that AEF retains its biological activity over a considerable length of time [52]. Notably, AEF is also effective when administered orally and can be serially transferred among animals. Furthermore, treatment of AEF with denaturing agents completely abrogates its activity [52]. These findings come tantalizing close to the characteristics of infectious prion proteins. An important difference, however, is that injecting AEF in the absence of an inflammatory stimulus does not lead to disease, and amyloid deposition and disease appear after inflammation even in the absence of AEF inoculation [48]. Therefore, analogous to the results in transgenic mice models of Alzheimer's disease, the phenomenon cannot be classified as infectious, but rather as an acceleration of the disease process.

Mouse senile amyloidosis

In mice, ApoAII amyloid accumulates in diverse organs during aging, leading to senile amyloidosis [53]. Although young mice do not accumulate apoAII fibrils, Higuchi *et al.* [54] found that a single intravenous injection of a very small amount of the apoAII fibrils induced severe systemic deposition of amyloid in young mice. After injection, amyloid deposition occurred rapidly and advanced in an accelerated manner, as observed in spontaneous senile amyloidosis in mice. Strikingly, injection of denatured apoAII fibrils, native nonfibrillar apoAII contained in high-density lipoprotein particles, or denatured apoAII monomer did not induce amyloidosis [54].

In a follow up study, the same group showed that oral administration of apoAII amyloid fibrils for 5 consecutive days resulted in all mice developing amyloid deposits at 2 months of age. The plaques were located in the small intestine initially, but extended to the tongue, stomach, heart and liver 3–4 months after administration [55]. Notably, amyloid deposition was observed in young mice raised for three months in the same cage as old mice that had severe amyloidosis. ApoAII was found to be present in the feces of old mice, and induction of amyloidosis by injecting a fraction of their feces indicated that the propagation of amyloidosis among mice in the same cage probably occurred through the consumption of feces [55]. Interestingly, transmission of apoAII amyloidosis shows a 'strain phenomenon' analogous to the prion strains [56].

Taken altogether, the evidence is compelling that some of the proteins associated with other amyloid-related disorders can have prion-like infectious properties *in vivo*. Whether or not some of these diseases have an infectious origin under non-experimental conditions remains to be studied.

Concluding remarks

The important role of protein misfolding and aggregation in various human diseases has been clearly established in the past decade. Some of the most compelling studies come from TSEs, the only member of this group of diseases in which the pathology is naturally and experimentally transmitted among individuals by administration of the misfolded protein. The molecular mechanism underlying prion propagation is strikingly similar to the mechanism of amyloid formation, which suggests that disease propagation by a protein infectious agent might be more common than we currently think. In addition, recent reports have provided intriguing evidence for the experimental transmission of other PMDs. An important challenge for the coming years will be to evaluate whether or not the prion mechanism of disease transmission might be operating in some of the most prevalent human diseases.

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