New Type of Prion May Cause And/Or Transmit Rare But Fatal Brain Disorder

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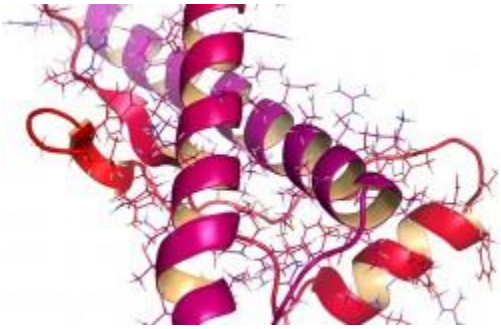

New Review Focuses on Parkinson's and Alzheimer's Diseases And Their Link to Prions

According to two new research papers led by University of California at San Francisco (UCSF) scientists, Multiple System Atrophy (MSA), a progressive neurodegenerative disorder with similarities to Parkinson's disease, is caused by a newly discovered type of prion, a variant of the misfolded proteins associated with incurable progressive brain diseases such Creutzfeldt-Jakob Disease (CJD or "mad cow disease"), that may be transmissible on contact.

First described in 1960, MSA is described by the National Institute of Neurological Disorders and Stroke as characterized by a combination of symptoms that affect both the autonomic nervous system (the part of the nervous system that controls involuntary action such as blood pressure or digestion) and movement. Rare but more common than CJD: MSA annually affects three out of 100,000 people over the age of 50, Symptoms of MSA reflect progressive loss of function and death of different types of nerve cells in the brain and spinal cord, with early-stage symptoms that can be mistaken for those of Parkinson's disease, including movement and balance problems, loss of bladder control, and disruption of blood-pressure regulation and other functions governed by the autonomic nervous system. However, unlike Parkinson's patients, who often live 10 to 20 years after being diagnosed, MSA patients typically die within five to 10 years and do not respond to the drugs or deep brain stimulation used for Parkinson's symptoms.

The UCSF researchers' findings suggest new approaches to developing treatments for MSA, for which there are currently no treatments to delay the progressive neurodegeneration and no cure, but also highlights a potential concern for clinicians or scientists who come in contact with MSA tissue.

A UCSF release authored by freelance science writer Nicholas Weiler notes that the new findings mark the first discovery of a human disease caused by a new prion in 50 years, since work at the National Institutes of Health in the 1960s showed that human brain tissue infected with CJD could transmit neurodegeneration to chimpanzees.
In 1982 UCSF professor of neurology and biochemistry Stanley Prusiner, MD, discovered an unprecedented class of pathogens that he named prions — "infectious proteins" — that cause neurodegenerative diseases in animals and humans. Dr. Prusiner found that prions were the causative agent for a brain-wasting disease found in sheep called scrapie, subsequently determining that the same prion protein caused bovine spongiform encephalopathy (BSE), or "mad cow" disease, in cattle, and so-called "variant" Creutzfeldt-Jakob Disease in humans who had consumed BSE-contaminated beef or other animal tissues. that manifest as (1) sporadic, (2) inherited and (3) infectious illnesses.

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When proposed, many scientists considered Dr. Prusiner's concept of "infectious proteins" as well as his proposal that a a simple protein could possess multiple biologically active shapes or conformations and replicate and spread disease to be "heretical," contradicting as it did a tenet of modern biology that maintained only viruses and living microbes such as bacteria had the capacity to transmit disease. However subsequent research by Dr. Prusiner and others led to better understanding of how prions function at a molecular level, and Dr. Prusiner was awarded the Nobel Prize in Physiology or Medicine for this work in 1997, for his discovery that these self-replicating misfolded proteins cause Creutzfeld-Jakob Disease and other related forms of neurodegeneration.

Dr. Prusiner is now director of the Institute for Neurodegenerative Diseases at UCSF, and his contributions to scientific research have also been internationally recognized with numerous other prizes including the Potamkin Prize for Alzheimer's Disease Research from the American Academy of Neurology (1991); the Richard Lounsbery Award for Extraordinary Scientific Research in Biology and Medicine from the National Academy of Sciences (1993); the Gairdner Foundation International

Award (1993); the Albert Lasker Award for Basic Medical Research (1994); the Wolf Prize in Medicine from the State of Israel (1996); and the United States Presidential National Medal of Science (2009). He is a member of the National Academy of Sciences, the Institute of Medicine, the American Academy of Arts and Sciences and the American Philosophical Society, and a foreign member of the Royal Society, London.

Based on his seminal discovery that prions can assemble into amyloid fibrils — peptide or protein aggregates which assemble to form insoluble fibers that are resistant to degradation, plaques of which are found in brain tissue of Alzheimer patients and are associated with neurodegeneration, Dr. Prusiner proposed that the more common neurodegenerative diseases including Alzheimers and Parkinsons diseases may be caused by prions, with evidence continuing to accumulate that prions cause not only these common degenerative diseases, but also Amyotrophic Lateral Sclerosis (ALS, or Lou Gehrig's Disease) the frontotemporal dementias (FTDs), chronic traumatic encephalopathy (CTE) and multiple system atrophy (MSA). Much of Dr. Prusiner's current research is focused on developing therapeutics that reduce the levels of the specific prions responsible for Alzheimer's, Parkinson's, MSA, the FTDs, CTE and CJD.

"Now we've conclusively shown that a new type of prion causes MSA," UCSF's http://ind.universityofcalifornia.edu/aboutus/faculty/gilesk Kurt Giles, DPhil, associate professor of neurology, IND researcher and senior author on the second of the two new studies tells Mr. Weiler in the UCSF release. "This is our mark in the sand."

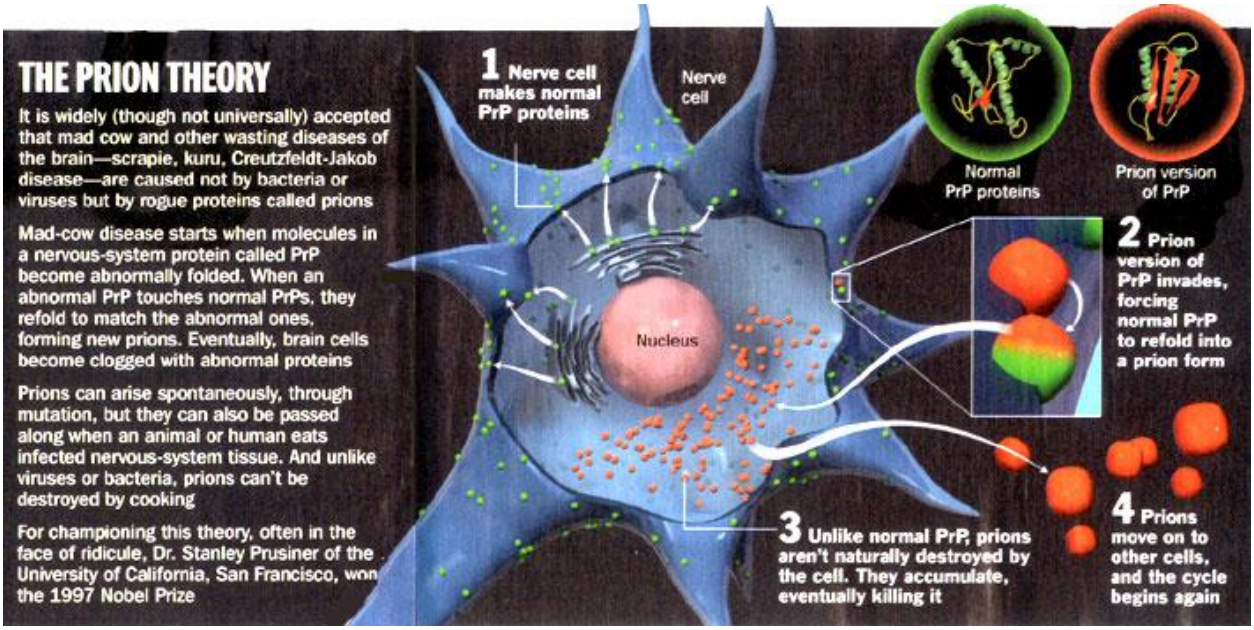
Mr. Weiler notes that, the original prion protein identified by Dr. Prusiner as being responsible for CJD, known as PrP, can exist in two forms: one of which harmless and the other fatal. PrP prions in the dangerous, misfolded form latch on to other nearby PrP molecules, causing them to lose their normal shape and initiating a chain reaction that results in sticky, insoluble plaques throughout the brain that kill off cells and result in the typical "spongy" appearance of CJD-affected brains — hence the designation "spongiform".

In the new research papers, published the weeks of Aug. 17 and Aug. 31, in the Proceedings of the National Academy of Sciences, Prusiner, Giles, post-doctoral researcher Amanda Woerman, PhD, and an international team of colleagues report that a misfolded version of a protein called alpha-synuclein seems to act in a similar way to transmit MSA from diseased human brain tissue to mice and to human cell cultures.

A new research paper published in the journal Proceedings of the National Academy of Sciences, entitled "Evidence for a-synuclein prions causing multiple system atrophy in humans with parkinsonism" (Published online before print PNAS August 31, 2015, doi: 10.1073/pnas.1514475112), coauthored by Dr. Prusinera with an international team of investigators including researchers from UCSF; Daiichi Sankyo Co., Ltd.; the University of Texas Southwestern Medical Center; Imperial College London; Massachusetts General Hospital; Stanford University; UC Los Angeles, and the University of New South Wales. describes how experiments conducted in Dr. Prusiner's lab in 2013 revealed that samples of brain tissue from two human MSA patients were able to transmit the disease to a mouse model for Parkinson's disease, expressing a mutant human alpha-synuclein gene. To confirm this finding, Prusiner and colleagues expanded this experiment to include tissue samples from a dozen more MSA victims from tissue banks on three continents: the Massachusetts Alzheimer's Disease Research Center in Boston, the Parkinson's UK Brain Bank at Imperial College London, and the Sydney Brain Bank in Australia.

The coauthors note that MSA is caused by a different human prion composed of the -synuclein protein, and while brain extracts from Parkinson's disease (PD) patients have not been found to be transmissible to genetically engineered cells or mice, they've discovered that -synuclein prions that cause the more common MSA), but that brain extracts from 14 MSA cases all transmitted neurodegeneration to mice. When exposed to human MSA tissue, the mice developed neurodegeneration. In addition, the team found that the brains of infected mice contained abnormally high levels of insoluble human alpha-synuclein, and that infected mouse brain tissue could itself spread the disease to other mice, observing that -synuclein is the first new human prion to be identified, to the investigators' knowledge, since the discovery a half century ago that CJD was transmissible.

Mr. Weiler notes that the discovery that alpha-synuclein prions can transmit MSA raises a public health concern regarding treatments and research that involve contact with brain tissue from neurodegeneration patients, because standard disinfection techniques that kill microbes do not eliminate the PrP prions that cause CJD, although whether the same challenges hold for alpha-synuclein prions in MSA remains to be determined.



In the meantime, PNAS paper's coauthors advise that clinicians and researchers should adopt much more stringent safety protocols when dealing with tissue from patients with MSA and other neurodegenerative diseases, many of which they believe may also be caused by prions. For instance, MSA is frequently initially misdiagnosed as Parkinson's disease, which is often treated with deep-brain stimulation with potential for the disease to be transmitted to other patients if deep-brain stimulation equipment is reused, even with standard sterilization protocols in place.

"You can't kill a protein," Dr. Giles observes in the release. "And it can stick tightly to stainless steel, even when the surgical instrument is cleaned." As a result, he said, "We're advocating a precautionary approach. People are living longer and likely getting more brain surgeries. There could be undiagnosed neurodegenerative diseases that – if they're caused by prions – mean infection could be a real worry."

However, unlike the danger of BSE from contaminated beef, the researchers stress that there is no apparent risk of infection by MSA prions outside of specialized medical or research settings.

In another PNAS paper published earlier in August entitled "Propagation of prions causing synucleinopathies in cultured cells" (PNAS August 18, 2015 Published online before print doi: 10.1073/pnas.1513426112), Dr. Amanda L. Woerman of the UCSF Institute for Neurodegenerative Diseases led a research team in development of a rapid new method to test prion transmission using human cell cultures, and was able to demonstrate that it only takes four days for human MSA tissue to infect cultured cells with alpha-synuclein mutations, in contrast to the 120 days it takes for the disease to spread to mouse models. The investigators conclude: "Our studies should facilitate investigations of the pathogenesis of both tau and a-synuclein prion disorders as well as help decipher the basic biology of those prions that attack the CNS."

"The challenge of studying neurodegeneration is that it's a disease of aging," Dr. Woerman told Mr. Weiler. "You have to let the mouse models develop for such a long time that research on cures is really slow to progress. Now, with these cell models, we can test how to inactivate alpha-synuclein aggregates at a speed that just wouldn't be feasible in animals."

Mr. Weiler notes that the UCSF researchers are working with Japanese pharmaceutical company Daiichi Sankyo, as part of a collaboration established in 2014 to develop potential treatments for prion diseases.

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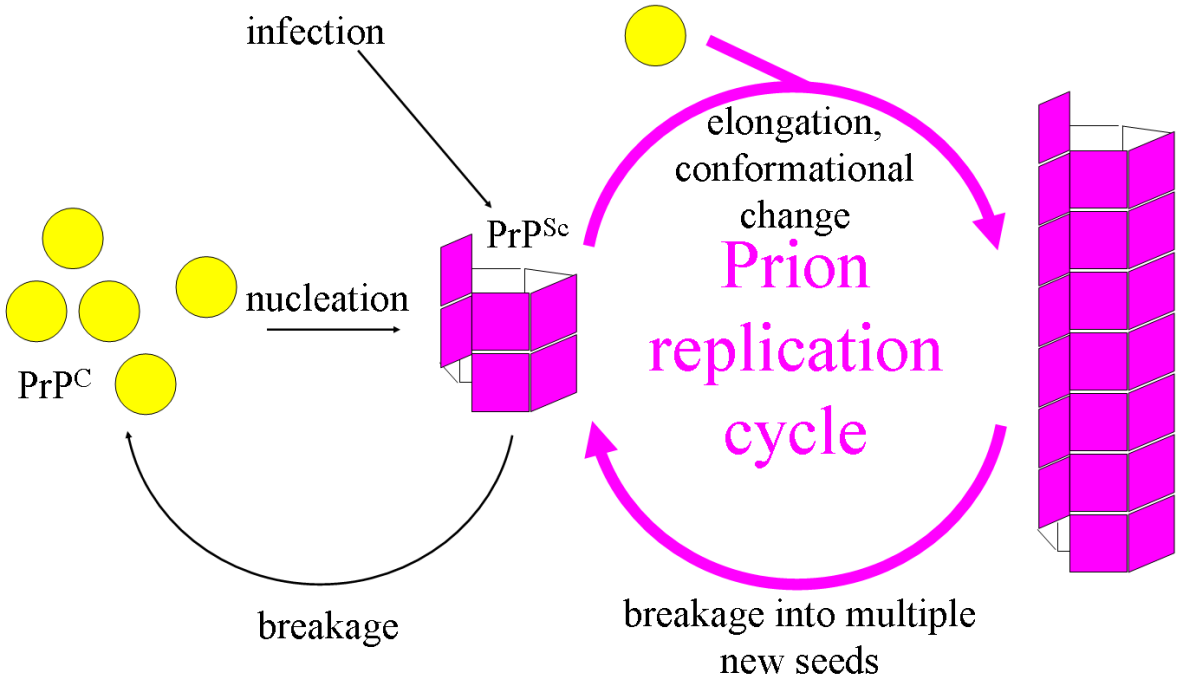
Sources:
University of California at San Francisco (UCSF)
UCSF Institute for Neurodegenerative Diseases
Proceedings of the National Academy of Sciences
National Institute of Neurological Disorders and Stroke



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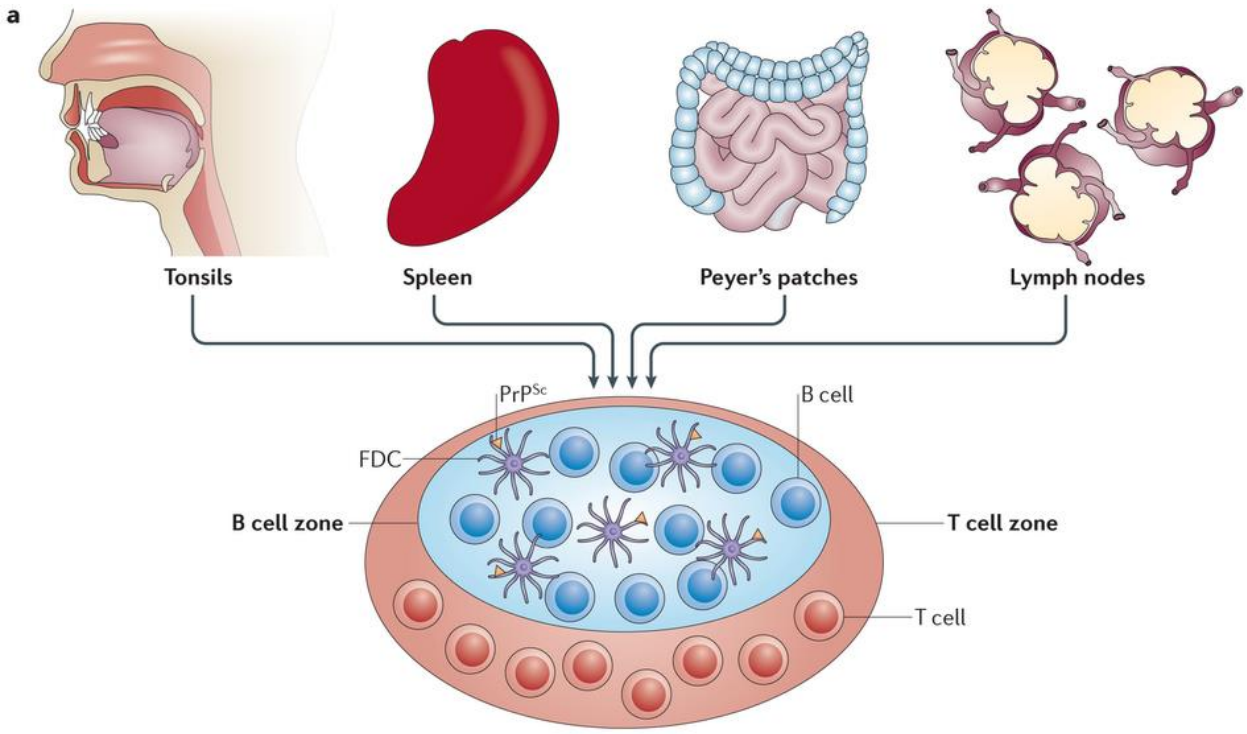

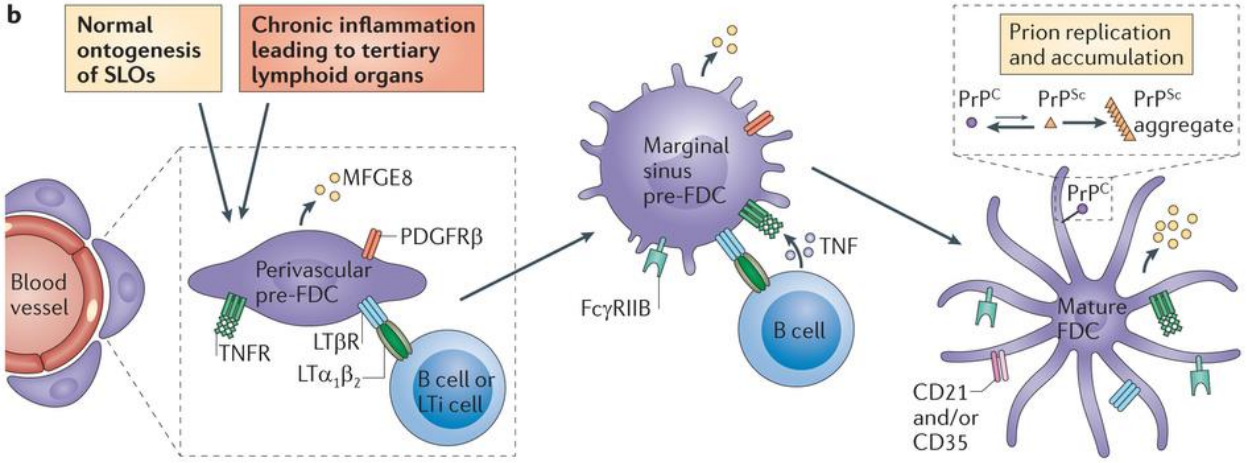

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Prion hypothesis: the end of the controversy?

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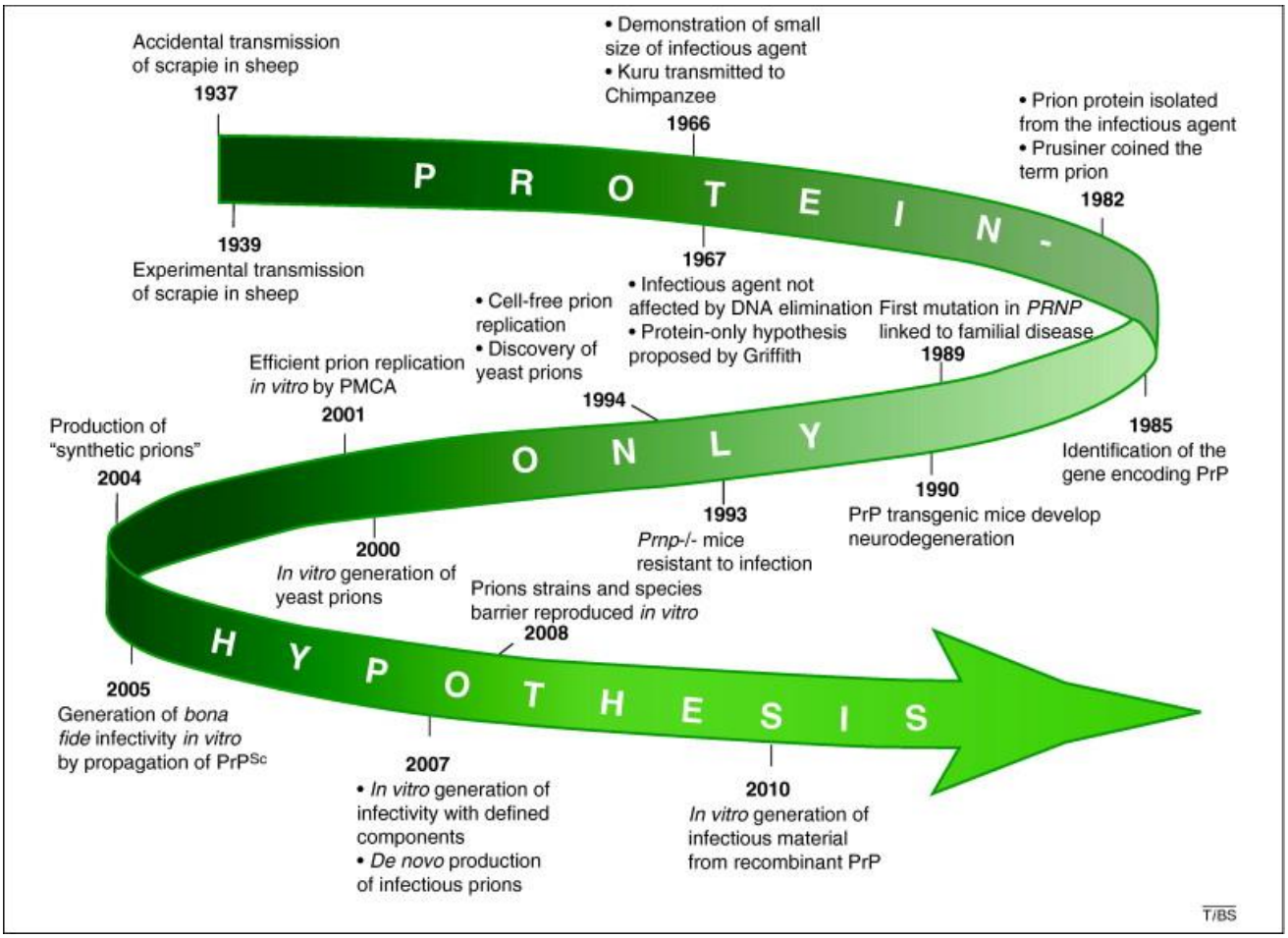

Figure 1

A timeline representation of the major milestones in the prion hypothesis. Starting with the initial indication that prion diseases can be transmissible, owing to the accidental transmission of scrapie in sheep, and ending with the demonstration that infectious material can be generated *in vitro* using pure recombinant prion protein, the prion field has always been full of unorthodox discoveries.

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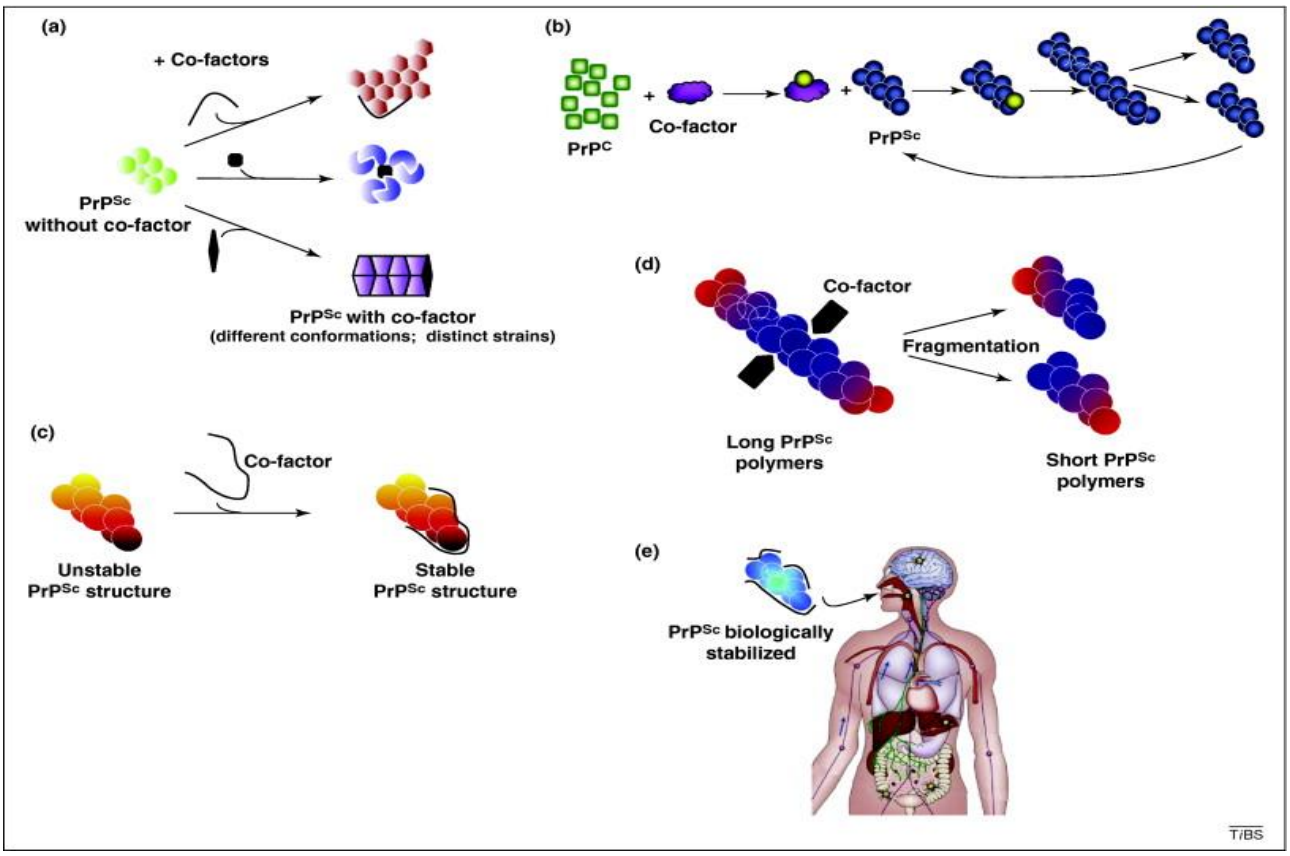

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The mysterious prion diseases

Prion diseases are a group of fatal and infectious neurodegenerative diseases that affect humans and diverse animal species [1]. The infectious nature of prion diseases was first evident more than 70 years ago when accidental transmission of scrapie occurred in sheep. Inoculation against a common virus with a formalin extract of tissue that was unknowingly derived from an animal with scrapie infected nearly 10% of the flock. Scrapie was subsequently transmitted experimentally to sheep [2] and later to mice [3]. In humans, an infectious origin was suspected for kuru, a prion disease identified in some cannibalistic tribes of New Guinea; a formal demonstration came in 1966 with the transmission of kuru to monkeys [4]. These studies were followed by transmission of Creutzfeldt–Jakob disease (CJD) [5] and Gerstmann–Straussler–Scheinker syndrome, an inherited form of prion disease, to primates [6]. These discoveries added to the initial findings that suggested that the infectious agent responsible for these diseases was different from any known form of microorganism, and led to a plethora of research that was aimed at understanding the nature of the agent. A second boost in the prion field came when an outbreak of bovine

spongiform encephalopathy (BSE) in cattle in the UK was shown to be mediated by prion infection [7]. Between the discovery of this disease as a prion disorder and the implementation of measures to prevent possible transmission to humans, it is likely that the entire population of the UK and a large part of the European population might have consumed infected meat. These initial concerns were confirmed when a new form of human disease, termed variant CJD, appeared and was convincingly linked to BSE [8, 9]. This article focuses on recent advances regarding the nature of the infectious agent that is responsible for prion diseases and the mechanisms of prion propagation.

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A historical perspective of the prion hypothesis

Starting from the initial discoveries in the prion field, it was clear that the infectious agent responsible for prion diseases was different from conventional microorganisms. Over decades of research, many milestone discoveries have provided crucial evidence in favor of the prion hypothesis, which proposes that a misfolded protein is the main (and perhaps the sole) component of this unorthodox infectious agent called a prion (Figure 1). In a series of key experiments, Alper and co-workers demonstrated that procedures that obliterate nucleic acids, such as very high doses of ionizing radiation and UV, do not destroy the infectious material [10, 11]. The same group also reported that the minimum molecular weight to maintain infectivity (~2×10^5 Da) is too small to be a virus or other type of microorganism [12]. This estimate is in the range of the size of most infectious prion particles that are obtained with modern sophisticated techniques [13]. Based on these observations, speculation arose that the infectious agent might be protein-based [14]. In 1967, Griffith proposed three scenarios by which a protein could act as the infectious agent that causes scrapie [15]. However, little research was done to test this hypothesis until Prusiner and coworkers pioneered an impressive set of discoveries that gave fuel to the prion hypothesis, and coined the term prion to refer to this heretical proteinaceous infectious agent [16].

In landmark research, the protease-resistant prion protein (PrP^{Sc}) was isolated from the infectious material [17]. PrP^{Sc} and infectivity co-purified, and the protein concentration was proportional to the infectivity titer [18]. These breakthroughs radically changed the prion field, and much effort then turned to study this protein. Infectivity was shown to be retained in highly purified preparations of PrP^{Sc}, in which no other protein was detectable, although the sample did contain lipids and carbohydrates, and perhaps nucleic

acids. Importantly, infectivity is reduced by agents that destroy protein structure, and strikingly, by anti-PrP antibodies [18]. The gene that encodes PrP (*PRNP*) was identified in the mammalian genome [19, 20], and the corresponding mRNA was shown to be the product of a single host gene, which is expressed mostly in the brain, without significant changes in expression in healthy or infected animals. These findings indicated that the prion protein can exist as both a normal cellular protein (termed PrP^{C}) and a pathological isoform (termed PrP^{Sc}) [21]. Surprisingly, no chemical differences can be detected between the two forms of the protein; rather, the nature of their distinct properties appears to be enciphered in the conformation adopted by the proteins [22]. Once *PRNP* was identified, genetic analyses discovered the first *PRNP* mutation linked to familial prion disease [23]; subsequent studies have shown that all of the familial cases of prion disease are linked to *PRNP* mutations [1]. Moreover, overexpression of mutant *Prnp* in mice produces a transmissible neurodegenerative disorder similar to prion disease [24,25, 26, 27, 28]. Strong evidence in favor of the prion hypothesis has come from the demonstration that $Prnp^{-/-}$ mice are resistant to scrapie infection, and do not develop signs of the disease, or allow propagation of the infectious agent [29]. The cell-free conversion of PrP^{C} into PrP^{res} that is catalyzed by the pathological protein also provides support for the prion hypothesis. In this system, developed by Caughey and co-workers [30], purified PrP^{C} mixed with stoichiometric amounts of purified PrP^{Sc} produce a low yield of PrP^{res} formation under non-physiological conditions, which precludes infectivity studies. In 2001, we developed a new system for *in vitro* replication of prions, termed protein misfolding cyclic amplification (PMCA) [31]. PMCA reproduces prion conversion in a greatly accelerated and efficient manner [31]. The *in vitro*-generated material maintains all the biological, biochemical and structural characteristics of *in-vivo*-produced prions [32]. These findings have confirmed a central tenet of the prion hypothesis, which is that prions can propagate indefinitely and that newly generated PrP^{Sc} triggers further misfolding to produce an auto-catalytic process of prion replication.

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Is the prion hypothesis proven?

Despite the compelling evidence for the prion hypothesis, skeptics have argued that definitive proof cannot be achieved until infectious material is produced *in vitro* from pure, normal prion protein [33]. Such an experiment remained elusive for many years. However, in 2004 the production of ‘synthetic prions’ via the *in vitro* induction of misfolding and aggregation of bacterially expressed recombinant prion protein

(rPrP) was reported [34]. Injection of this material into transgenic mice that overexpress PrP induces a transmissible neurodegenerative disease with a very long incubation period. The generated infectious material displays different characteristics compared to those of natural prions. The authors proposed that conformational adaptation must have occurred to stabilize the originally extremely low infectivity of misfolded rPrP, which paradoxically involves a decrease in the thermodynamic stability of the aggregate [35]. These findings and subsequent studies from the same group [35, 36, 37], including the formation of many novel synthetic prions and even some composed of protease-sensitive PrP, have come very close to providing the definitive proof for the prion hypothesis. Unfortunately, the fact that the disease is transmissible only to transgenic animals that overexpress PrP, and not to wild-type animals, is a matter of concern, given the well-known propensity for such animals to develop a prion-like disease spontaneously [38, 39, 40]. In other words, the disease in these animals might have resulted by acceleration, induced by the injected material, of a pre-existing condition produced by transgenesis, as is the case in transgenic mice that overexpress mutant PrP [28]. Recent work using a different protocol to induce misfolding and aggregation of rPrP has generated infectious prions after several passages [41]. Importantly, this study demonstrated induction of disease in wild-type animals.

PMCA has proven to be a very successful technique for generation of *bona fide*infectious prions *in vitro*. Using PMCA, our group generated prions *in vitro* that were infectious to wild-type animals and displayed similar properties to brain-derived infectious material [32]. PMCA has become an invaluable technique to study prions, given that it provides the equivalent to a procedure for cultivating prions *in vitro*. In an extension of this work, Supattapone and colleagues generated infectious material using PMCA and PrP^{C} and PrP^{Sc} purified from the brain of healthy and sick animals, respectively [42]. Infectivity was generated in the absence of any other component besides the purified proteins and a synthetic polyanion that was used to catalyze the reaction. Importantly, this study, as well as a recent one from our group, showed that PMCA can generate*de novo* infectious PrP^{Sc} in the absence of any infectious brain-derived material to begin the reaction [42, 43]. When spontaneously generated prions are injected into wild-type animals, the disease exhibits unique clinical, neuropathological and biochemical characteristics that differ from those of all previously known prions. An important question to be answered by future research is whether the *de novo*prions are present in the normal brain or are generated during the amplification process. A recent study that has shown the spontaneous generation of prions amplified by prion

replication in cells also highlights this important issue [44]. In this study, infectivity was detectable at low frequency after incubation of uninfected mouse brain homogenate with metal wires that presumably concentrate prions. These remarkable findings lend strong support to the prion hypothesis, and provide a mechanistic explanation for the sporadic form of prion diseases. Moreover, they suggest that new forms of prions with different disease characteristics will probably appear in the future. All these PMCA studies were performed using mammalian PrP^{C} as a substrate. Formation of protease-resistance PrP from protein recombinantly produced in bacteria, seeded by brain PrP^{Sc}, was first achieved by Atarashi *et al*., by using an adapted version of PMCA [45]. Although material generated in this way is infectious to wild-type animals, it requires a long incubation period and results in an incomplete attack rate [46], which suggests that purified rPrP, in the absence of other cellular factors, is not capable of producing a highly infectious preparation. However, in a recent and elegant study, Wang *et al.* reported the *de novo* generation of *bona fide* infectious prions *in vitro* by PMCA using exclusively rPrP with the sole addition of RNA and lipids [47]. This study might represent the strongest proof to date for the prion hypothesis.

One argument that has often been used against the prion hypothesis is the existence of prion strains [48]. Nearly all transmissible spongiform encephalopathies are known to exhibit various strains that are characterized by different incubation periods, clinical features and neuropathology [49]. For infectious diseases, different strains generally arise from mutations or polymorphisms in the genetic make-up of the infectious agent. To reconcile the infectious agent that is composed exclusively of a protein with the strain phenomenon, it has been proposed that PrP^{Sc} obtained from different prion strains has slightly different conformation or aggregation states that can faithfully replicate at the expense of the host PrP^{C} [50, 51, 52]. This idea is supported by recent studies that have shown that a group of polythiophene compounds can differentially interact with PrP^{Sc} associated with distinct strains, thereby enabling them to be structurally distinguished [53]. Additional support for the hypothesis that the strain features are enciphered in the structure of PrP^{Sc} was provided by our recent study that has shown that PMCA can faithfully replicate different prion strains in humans and mice, which maintain all their features after multiple passages *in vivo* and *in vitro* [54]. Furthermore, the related phenomena of species barrier, strain adaptation, and molecular memory have also been reproduced *in vitro* by PMCA [55, 56, 57], which suggests again that they are dependent purely on PrP^{Sc} replication. When PrP^{C} from one species is used

to replicate prions from a different species, new strains are generated, which points to an extremely high flexibility of PrP and raising the possibility of infinite ‘prionability’ of the protein.

Overall, the impressive recent progress in production of infectious material has removed all doubts about the prion hypothesis. In addition, the demonstration that several other proteins in a variety of organisms (such as yeast, fungi and invertebrates) use the prion mechanism to transmit biological information has strengthened the prion hypothesis (Box 1). Based on the available data, the idea that prions consist of viruses or any other type of conventional microorganism is simply untenable. Nevertheless, several key aspects of PrP^{Sc} infectivity remain unknown; particularly the detailed structure of the infectious protein, and whether or not another element besides the prion protein is required for prion replication.

+

Box 1

Yeast prions

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Mechanism of prion replication and potential roles of co-factors in prion infectivity

Prion replication begins when PrP^{Sc} in the infectious material interacts with host PrP^{C}, thereby catalyzing its conversion to the pathogenic form of the protein. The precise molecular mechanism of $PrP^{C} \rightarrow PrP^{Sc}$ conversion is not completely understood, but available data support a seeding/nucleation model in which infectious PrP^{Sc} is an oligomer that acts as a seed to bind PrP^{C} and catalyze its conversion into the misfolded form by incorporation into the growing polymer [58, 59, 60]. At some point, the long PrP^{Sc} polymers break into smaller pieces driven by a mechanical force or catalyzed by a yet-unknown process. This fragmentation increases the number of effective nuclei that can direct further conversion of PrP^{C}. Polymer fragmentation is probably a rate-limiting event in prion replication, therefore, it is important to understand how this occurs*in vivo*, and whether another cellular factor participates in this process. The seeding–nucleation model provides a rational and plausible explanation for the infectious nature of prions. Infectivity relies on the capacity of preformed stable misfolded oligomeric PrP^{Sc} to act as a seed to catalyze the misfolding and aggregation process [60]. Indeed, the PMCA technology is based on the

assumption that prion replication depends on the formation and multiplication of oligomeric seeds [31, 59].

An open question in the field is whether or not prion replication requires a cellular co-factor. Several pieces of evidence indicate that co-factors might participate in prion replication [61]. The existence of a host-encoded conversion factor was first suspected from experiments with transgenic mice that expressed chimeric prion proteins from two different species [62]. Based on these findings, Prusiner coined the term ‘protein X’ to refer to this factor; however, there is no formal proof that the accessory molecule is indeed a protein. Further evidence for the existence of conversion factors came from PMCA studies of $PrP^{C} \rightarrow PrP^{Sc}$conversion. Purified hamster PrP^{C} is not converted when mixed with highly purified PrP^{Sc}; however, conversion is restored when the complete brain homogenate is added to the sample [63, 64, 65]. These results suggest that unknown factors present in brain homogenate are essential for prion conversion. Supattapone's group has shown that natural or synthetic RNA can act as conversion factors and catalyze prion replication in hamsters [66], but surprisingly, not in mice [67]. It has been proposed that RNA might serve as a scaffold for efficient conversion and a necessary molecular complex-pair as the minimal infectious unit [68]. These data suggest that nucleic acids might be, after all, involved in prion replication. However, an alternative possibility is that RNA only accelerates or stabilizes an otherwise experimentally inaccessible PrP^{Sc}-like conformation. The same group has shown that it is the negative charge of RNA that is responsible for the interaction with PrP, given that several other natural or synthetic polyanions can have the same activity [64]. These results, however, do not fit with the initial finding that only eukaryotic RNA, and not prokaryotic RNA, assists prion conversion [66]. More recent studies have shown that replication of PrP^{Sc} and infectivity that stems from rPrP require synthetic anionic phospholipids in addition to RNA [47]. The requirement for lipids is consistent with previous studies that have reported higher infectivity with lipid-membrane-associated PrP^{Sc} [69, 70]. In spite of these findings, it is possible that RNA and lipids are merely *in vitro* mimics of the activity of one or more unknown *in vivo* facilitating factors. Recent attempts to identify the mysterious co-factor, using a complementation PMCA assay and fractionation techniques, have shown that it is present in all major organs of diverse mammalian species, and is predominantly located in the lipid raft fraction of the cytoplasmic membrane [65]. However, conversion factor activity is not present in the lower organisms tested (yeast, bacteria and flies), which suggests that prion replication is a feature that is only present in

mammals. Surprisingly, treatments that eliminate nucleic acids, proteins or lipids do not prevent prion replication *in vitro* [65]. One possible interpretation of these findings is that various different compounds might act as a conversion factor *in vitro*, such that elimination of only some of them does not prevent prion replication. Indeed, the addition of various classes of molecules (synthetic nucleic acids, heparin, albumin or fatty acids) produces a small but detectable effect on enhancing prion replication *in vitro* [65].

If co-factors are indeed necessary for prion replication, it is possible to imagine at least five possible roles played by these accessory molecules (Figure 2):

- (i)

  The co-factor might contribute biological information to the infectious material. If the accessory molecule is incorporated into the PrP^{Sc} particles, it is possible that it could help to determine the folding characteristics of prions. In addition, it might facilitate the interaction with cellular components and modify the tropism of the infectious agent. In this case, the co-factor could have an important role in modulating the properties of prion strains. A recent study provided indirect evidence for this possibility [71]. Experiments using a panel of cells to replicate mouse prions have shown that infection of different cell types leads to phenotypic “mutation” and selection of prion strains. One possible explanation for these results is that cellular diversity in co-factors could direct prion strain changes, given that no differences in PrP were detected between the different cells [72].
- (ii)

  The co-factor might act as an essential catalyst for prion replication. The conversion factor could facilitate the PrP^{C} to PrP^{Sc} conversion, perhaps by directing the interaction of the two proteins, or through binding to PrP^{C} and inducing a partial unfolding of the protein that is necessary to adopt the misfolded conformation. The idea that co-factors facilitate prion replication is supported by *in vitro* conversion experiments that have shown that the PrP^{C} to PrP^{Sc} conversion is more efficient in the presence of accessory molecules [47,65, 67].
- (iii)

  The co-factor might help to stabilize the conformation of PrP^{Sc}. In this model, although the co-factor does not directly participate in the conformational conversion, it might contribute to stabilization of the newly formed misfolded structure. This view is supported by neuropathological data, especially from other neurodegenerative diseases, which show that attachment of a variety of molecules (e.g. proteins, nucleic acids, metal ions, or proteoglycans) to cerebral protein aggregates makes them much more compact and stable structures [73,74, 75].

- (iv)

  The co-factor might participate in the fragmentation of PrP^{Sc} polymers. As described above, prion replication follows a seeding–nucleation process of polymerization. For this process of seeding–nucleation to propagate prions efficiently, it is necessary not only for PrP^{Sc} to be able to seed the misfolding and aggregation of PrP^{C}, but also for long polymers to be fragmented into smaller pieces as a way to increase the effective number of seeds that catalyze prion replication. Indeed, in yeast prions (Box 1), the chaperone heat shock protein 104 (Hsp104) probably fulfills this role [76, 77], given that the replication of most yeast prions depends on this factor and that inhibition of Hsp104 activity precludes prion propagation [78].

- (v)

  The co-factor might increase the biological stability of prions, thereby reducing their *in vivo* clearance. Another possibility is that the co-factor increases the pharmacokinetic and bioavailability properties of PrP^{Sc}. In this sense, it is important to emphasize that the ability of an infectious agent to propagate disease depends not only on its successful replication, but also on its ability to remain intact in the body and reach the target location. This is a challenging task for an infectious agent that is composed solely of a protein. Recent studies have identified a possible pathway for prion clearance that involves milk fat globule-EGF factor 8 protein (MFGE8)-mediated phagocytosis of prions by microglia [79]. In this context, a co-factor could reduce the affinity of PrP^{Sc} for MFGE8, thereby decreasing the ability of microglia to engulf and destroy prions. The possibility that the co-factor contributes to the biological stability of PrP^{Sc} is in agreement with results that have shown that attachment of prions to nitrocellulose paper, soil particles or steel wires increases prion infectivity [80, 81, 82].

One of the most important issues related to the existence of possible components other than PrP that are involved in prion transmission is to distinguish between factors contained in the infectious particle and host factors that are involved in the conformational change. This distinction is very relevant; if additional factors must be part of the infectious particle, then the infectious units would not be composed solely of PrP^{Sc}. Alternatively, additional factors might need to be present in the host cells to sustain efficient prion replication. These factors could be normal cellular components that are presumably engaged in other functions in the infected cells, which accidentally participate in prion conversion. In the latter case, these additional factors should not be considered part of the infectious particle, but rather host-encoded molecules that facilitate prion replication.

Jump to Section

Concluding remarks

The hypothesis that a single protein is the sole component of the infectious agent responsible for prion diseases has been highly controversial for the past 30 years. This is not surprising considering that the prion hypothesis claims that a protein behaves like a living microorganism to infect an individual by various routes (even oral administration), survive metabolic clearance, self-replicate in the body, reach the target organ, and induce a cascade of neurodegenerative damage, which results in disease and ultimately death of the infected individual. Moreover, the prion agent exists as multiple strains that lead to diseases with sometimes subtle and other times overtly different clinical and neuropathological characteristics. Prion propagation also respects the still incompletely understood rules of species barriers and exhibits characteristics of strain adaptation and memory. All of these features can be easily explained by an agent that contains genetic material, but a protein-based infectious agent makes an explanation more challenging. For this reason, although compelling evidence has accumulated steadily to support the prion hypothesis, the majority of scientists have remained skeptical. However, exciting research in the past 3 years has demonstrated that infectious material can be generated with defined components *in vitro*, and that the information for strains and the species barrier appears to be exclusively contained in the folding of PrP^{Sc}. These findings have proven beyond any doubt that the prion hypothesis is indeed correct. This does not mean that everything is known regarding prions. On the contrary, there are many outstanding questions still unanswered (Box 3). Among these, it is particularly important to understand whether or not other cellular factors play a role in prion replication. This is not simply a matter of curiosity; it is crucial to understand the nature of the infectious agent. In this context we are still uncertain whether the prion hypothesis is equivalent to a PrP^{Sc}-only hypothesis. Although it is clear that PrP^{Sc} is the main informational molecule in the infectious agent, it remains possible that a co-factor is required, perhaps to achieve efficient propagation in the *in vivo* setting. In the latter scenario, a molecule other than PrP^{Sc} would be essential for infectivity. Nevertheless, the putative existence of a co-factor, and its potential role in the process, does not negate the protein-based mechanism of transmission by which prions produce disease. Another important area of research is to elucidate the 3D structure of PrP^{Sc} and determine the structural features of prion strains. The remarkable progress in the generation of infectious prions from purified components, owing to the successful application of the PMCA technique, opens the

possibility to produce large amounts of chemically homogeneous and metabolically labeled infectious PrP^{Sc}, which paves the way to fine structural studies.

The advances in the prion hypothesis have paralleled the expansion of the prion concept beyond prion diseases. Indeed, recent years have witnessed the end of a period of skepticism surrounding the possibility that a single protein could act as an infectious agent, and the beginning of an era in which the prion phenomenon is being expanded to some of the most prevalent human diseases (Box 2) [60, 83, 84, 85]. If further research proves that several other diseases can be transmitted by the propagation of misfolded proteins, prions could turn out to be more than a rare caprice of nature.