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Minireview

MINIREVIEW

A brief history of prions

Mark D. Zabel* and Crystal Reid

Prion Research Center at Colorado State University, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO 80521, USA

*Corresponding author: Prion Research Center at Colorado State University, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO 80521, USA. Tel: 001+(970) 491-1455; Fax: 001+(970) 491-0703; E-mail: mark.zabel@colostate.edu

One sentence summary: A fascinating tale of the journey that led to the discovery of infectious proteins as a new class of pathogens.
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ABSTRACT

Proteins were described as distinct biological molecules and their significance in cellular processes was recognized as early as the 18th century. At the same time, Spanish shepherds observed a disease that compelled their Merino sheep to pathologically scrape against fences, a defining clinical sign that led to the disease being named scrapie. In the late 19th century, Robert Koch published his postulates for defining causative agents of disease. In the early 20th century, pathologists Creutzfeldt and Jakob described a neurodegenerative disease that would later be included with scrapie into a group of diseases known as transmissible spongiform encephalopathies (TSEs). Later that century, mounting evidence compelled a handful of scientists to betray the prevailing biological dogma governing pathogen replication that Watson and Crick so convincingly explained by cracking the genetic code just two decades earlier. Because TSEs seemed to defy these new rules, J.S. Griffith theorized mechanisms by which a pathogenic protein could encipher its own replication blueprint without a genetic code. Stanley Prusiner called this proteinaceous infectious pathogen a prion. Here we offer a concise account of the discovery of prions, the causative agent of TSEs, in the wider context of protein biochemistry and infectious disease. We highlight the discovery of prions in yeast and discuss the implication of prions as epigenomic carriers of biological and pathological information. We also consider expanding the prion hypothesis to include other proteins whose alternate isoforms confer new biological or pathological properties.

Keywords: prion; protein; infectious disease; review

INTRODUCTION

Proteins were described as distinct biological molecules and their significance in cellular processes was recognized and reported as early as the 18th century by the french chemist Antoine Fourcroy (Tanford and Reynolds 2001; Perrett 2007). Nearly a century later, in 1838, Dutch chemist Gerhardus Johannes Mulder biochemically characterized this 'fundamental substance' (Tanford and Reynolds 2001) and Jons Jakob Berzelius named it 'protein' after the Greek word 'prota', meaning 'of primary importance'. Early theories explaining protein structure, folding and function were widely disputed, and predicting protein folding continues to challenge researchers today. Perhaps not surprising then, research into a protein that defies biochemical and biological paradigms like the prion protein reveals a fascinating and controversial story that challenges biological dogma and ultimately lends insight into protein biochemistry and bioinformation storage and transfer. This story also begins in the 18th century (Fig. 1), with unrelated observations of a strange disease affecting Merino sheep that caused abnormal behavior such as altered gaits, excessive licking and intense itching that compelled affected sheep to pathologically scrape against fences. Scrapie, as it was called, would later be designated as the first member of a new class of neurological disorders known as transmissible spongiform encephalopathies (TSEs).

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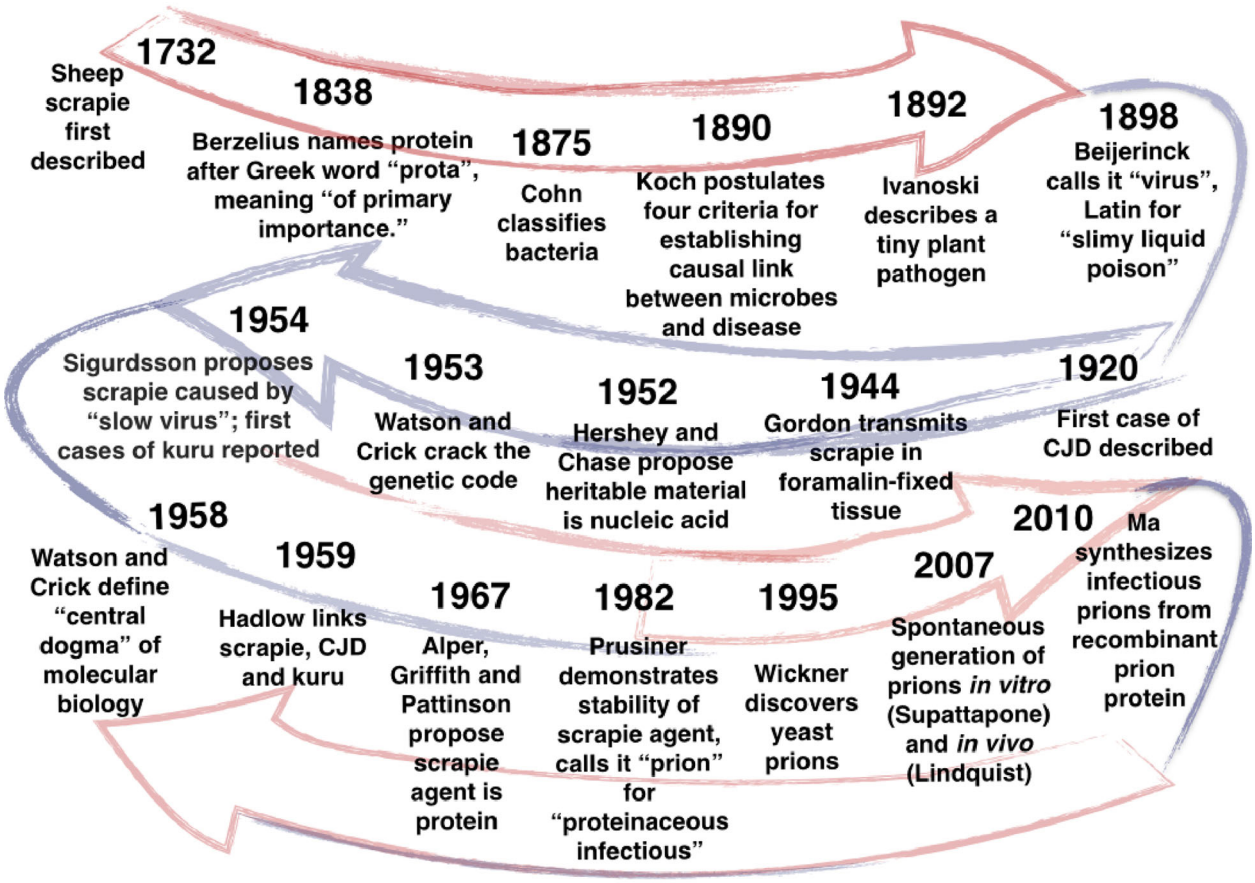

Figure 1. Significant events in the discovery of proteins, infectious disease and prions.

PATHOGENS, PROTEINS AND NUCLEIC ACIDS

In 1875, Ferdinard Cohn published an early classification of bacteria. Robert Koch reported that anthrax is caused by a bacterium a year later (Brock 1961). His Koch's postulates, published in 1890 and modified somewhat over the years, continue to guide microbiologists today to establish causal links between pathogens and disease. In 1891, Paul Ehrlich proposed that proteins that he termed 'antibodies' conferred immunity against pathogens (Piro et al. 2008). In 1892, D.I. Ivanoski was the first scientist to isolate and describe an infectious plant pathogen that was much smaller than any known bacterium (Lechevalier 1972). In 1898, M.W. Beijerinck confirmed Ivanovski's findings and named the filterable infectious pathogen contugium vivum fluidum, or a 'virus' (Lecoq 2001). Decades later, Wendell M. Stanley won a Nobel Prize for characterizing and crystallizing the tobacco mosaic virus, although, ironically, he initially mistook the virus for an infectious protein (Stanley 1935; Cohen and Stanley 1942). Hershey and Chase (1952) concluded through elegant experimentation that viral replication requires nucleic acid, the likely heritable material. A year later, James Watson, Francis Crick and Maurice Wilkins confirmed Hershey's and Chase's supposition and cracked the genetic code (Watson and Crick 1953). In 1958, Crick described the foundation of protein synthesis and defined what would soon be known as the 'central dogma of molecular biology': that information encoded by nucleic acid can be synthesized, stored and used by an organism to replicate itself (Crick 1970).

TEACHING OLD DOGMA NEW TRICKS: PROTEINS AS PATHOGENS AND BIRTH OF THE PRION

In 1920, neurologists Hans Gerhard Creutzfeldt and Alfons Maria Jakob described a human neurological disorder of unknown etiology (Creutzfeldt 1920; Jakob 1921) that would vex the scientific community for the next 60 years. Cuille and Chelle hypothesized that the sheep disease scrapie was caused by a 'slow virus' as early as 1938 (Cuille and Chelle 1938). Scientific revelations that viral nucleic acids encode genetic information and were infectious (Fraenkel-Conrat and Williams 1955; Fraenkel-Conrat 1956; Fraenkel-Conrat, Singer and Williams 1957) overwhelmingly directed the first theories of TSE etiology toward a 'slow virus' (Eklund, Kennedy and Hadlow 1967; Gajdusek 1967; Gajdusek and Gibbs 1968). Sigurðsson (1954) suggested that a slow virus caused sheep scrapie due to its long incubation period. Unfortunately, an important distinction between viruses and TSE pathogens was mistakenly overlooked 10 years earlier. In 1944, Veterinarian W.S. Gordon used formalin to inactivate louping-ill virus found in brain and spleen of infected animals. He then used these treated tissues to vaccinate healthy animals (Gordon 1946). Formalin inactivated the virus but not the scrapie agent that was unknowingly present, and the vaccinated animals died of scrapie two years later.

While Sigurdsson proposed that a slow virus causes scrapie, scientists discovered another human neurological disorder among the Fore tribe in Papua New Guinea called kuru that

presented much like Creutzfeldt–Jakob disease (CJD) and scrapie (Gajdusek and Zigas 1959). Like scrapie, kuru and CJD would not be categorized as a TSE for years even though clues to their infectious nature began to emerge much earlier. Inoculating brain and cerebral spinal fluid from scrapie-infected sheep into healthy ones transmitted disease (Plummer 1946; Fast and Groschup 2012). Collaborative efforts revealed in 1959 that kuru, scrapie and CJD were distinct forms of the same neuropathy (Hadlow 1959; Klatzo, Gajdusek and Zigas 1959). Hadlow proposed that kuru was transmissible like scrapie and he further anticipated, based on Sigurdsson's (1954) study, that kuru was a slow virus. A. G. Dickinson supported a role for nucleic acid in scrapie by identifying a gene that seemed to control scrapie incubation periods in some mouse strains (Dickinson, Meikle and Fraser 1968). Hadlow recommended infecting primates with brain material from humans that died of kuru. In 1966, Gajdusek, Gibbs and Alpers performed these experiments in chimpanzees and repeated them two years later with CJD brain material, revealing that both diseases were transmissible after intracerebral inoculation (Gibbs et al. 1968; Beck et al. 1969).

Two decades after Gordon failed to inactivate the scrapie agent with formalin, other scientists began to investigate scrapie stability. Many experts from different fields tried to inactivate the scrapie agent by ionizing and UV irradiation, extreme heat, high pressures and other compounds known to inactivate viruses and bacteria (Hunter and Millson 1964; Pattison 1965; Alper, Haig and Clarke 1966; Alper et al. 1967; Prusiner 1982). Characterization of the scrapie agent was more easily accomplished with mouse models (Chandler 1961, 1963). But these experiments may have distracted researchers from another piece of the puzzle that should have revealed striking differences between the scrapie agent and typical pathogens: earlier observations that CJD had a familial or genetic component of transmissibility (Meggendorfer 1930; Masters et al. 1979; Schoene et al. 1981).

A few prescient scientists including Tikvah Alper, I.H. Pattison and J.S Griffith speculated that the scrapie agent could be of protein origin. Their theories contradicted the central dogma of biology, the foundation for which Crick so elegantly provided just a few years earlier. Their attempts to experimentally inactivate the scrapie agent inspired their iconoclastic ideas. In 1966, Tikvah Alper attempted to use ionizing radiation to inactivate and determine the genomic size of the scrapie agent. She discovered thst the agent was not easily inactivated with high amounts of UV radiation and therefore, it must be replicating without nucleic acid (Alper et al. 1967). Pattison added further evidence that the scrapie agent was of protein origin based on his experiments designed to isolate it from formalin-fixed tissue (Pattison and Jones 1967). But J.S. Griffith was the first scientist to boldly speculate that the scrapie agent was proteinaceous. He also offered three mechanisms that might explain how a protein could be infectious and how this infection could be controlled genetically and occur spontaneously (Griffith 1967). Griffith alluded to the controversy that his hypotheses might engender in the discussion of his 1967 paper, '…the occurrence of a protein agent would not necessarily be embarrassing although it would be most interesting'.

Several researchers followed in Griffith's footsteps and accumulated data that continued to suggest that the scrapie agent was dependent upon protein (Hunter et al. 1969; Prusiner et al. 1978, 1980; Cho 1980; Merz et al. 1981). Stanley Prusiner, however, pushed the 'protein-only' hypothesis to a rebellious new level. Prusiner (1982) coined the term 'prion', proteinaceous infectious particle, to describe the infectious scrapie agent, for which he would later win the Nobel Prize. Prusiner *et al*. bolstered their prion hypothesis by isolating an infectious, proteinaceous amyloid from diseased animals and successfully inactivating the infectious agent contained therein by methods that destroyed proteins (Bolton, McKinley and Prusiner 1982; Prusiner et al. 1982a,b, 1983). Importantly, potent radiation and nucleases that destroy nucleic acids failed to inactivate prions.

THE PRION HYPOTHESIS: FOOL-PROOF OR A FOOL'S PROOF?

Despite Prusiner's bold attempts to prove the protein-only hypothesis, years passed before it was generally accepted by most of the scientific community. A.G. Dickinson investigated the genetic basis of prion disease in the 1960s to explain the phenomenon that certain mouse strains exhibited differential incubation periods when inoculated with the same scrapie brain homogenate (Dickinson and Mackay 1964). He used classical genetics to isolate a chromosomal locus in mice that controlled the incubation period of the ME7 strain of scrapie prions (Dickinson, Meikle and Fraser 1968). Dickinson named this locus sinc, for scrapie incubation.

Even if the scrapie agent is a prion, what is the source of this proteinaceous agent? Surely, a gene must encode the message that translates into the protein. In 1985, Bruce Chesebro and Richard Race searched for the origin of this message, and deduced an mRNA transcript from the protein sequence that encoded PrP 27–30, the protease-resistant prion particle isolated by Prusiner. Surprisingly, they found this prion protein mRNA in both infected and uninfected brain tissue (Chesebro et al. 1985; Locht et al. 1986). That same year Prusiner and Charles Weissman provided further evidence supporting the 'protein-only' hypothesis by discovering that a host cellular gene encodes the prion agent (Oesch et al. 1985). The infectious material lacked the gene encoding the cellular prion protein (PrP^C), a finding consistent with Prusiner's results from his inactivation experiments. George Carlson and Prusiner isolated the locus that contained the PrP^C gene and called it Prn-p. They found it to be closely linked to the sinc locus, which they called Prn-i. Inoculating scrapie-infected brain homogenate into mice genetically deficient in Prn-p failed to induce scrapie (Büeler et al. 1993), demonstrating the requirement of PrP^C for prion infection. Five years later, Jean Manson and Richard Moore performed elegant gene targeting experiments to demonstrate that Prn-p polymorphisms controlled incubation periods in prion-infected mice, providing strong evidence that Prn-p and Prn-i (sinc) are the same gene (Moore et al. 1998). Prions do not stimulate a humoral immune response, suggesting that the scrapie agent may be an immunologically inert host encoded protein (Prusiner et al. 1993), consistent with the prion hypothesis.

Genetic and biochemical spontaneous prion formation in vitro and in vivo provides the strongest evidence to date that prions are infectious, misfolded proteins that cause disease and lack instructional nucleic acid. Surachai Supattapone and Claudio Soto generated infectious prions de novo using a prion amplification technique and highly purified PrP^C from uninfected brain homogenate and synthetic polyanions (Deleault et al. 2005; Barria et al. 2009; Gonzalez-Romero, Morales and Soto 2009). Susan Lindquist created the human PRNP mutation that causes Gerstmann–Straüssler–Schenker (GSS) prion disease in mice, which developed spontaneous disease (Jackson et al. 2009). But Jiyan Ma conducted perhaps the most convincing experiment to date in support of the prion hypothesis: generation of infectious

prions from recombinant prion protein produced in bacteria (Zhang et al. 2013). These experiments represent the Holy Grail of prion research. Skeptics dismissed previous de novo prion generation experiments because they relied on extraction of PrP^{C} from living animals that might harbor a putative TSE virus. While some holdouts still favor a slow virus or even bacterial etiology of prion diseases (Manuelidis, Sklaviadis and Manuelidis 1987; Broxmeyer 2004; Bastian et al. 2007; Manuelidis 2007), most scientists now accept the premise that normal, host-encoded PrP^{C} can misfold into a pathologic form (PrP^{Sc}) to cause prion disease in susceptible hosts.

YEAST RAISE SUPPORT FOR PRIONS

The prion hypothesis received unexpected support from the simplest of eukaryotic organisms - yeast. Wickner (1994) discovered that a yeast nonchromosomal genetic element, [URE3], was an altered form of the yeast protein, Ure2p and proposed it to be a yeast prion. [URE3] permitted yeast to grow on poor nitrogen sources, specifically ureidosuccinate, the catabolism of which Ure2p represses. He also proposed the existence of a second yeast element, [PSI+], to be the prion form of the yeast prion Sup35. [PSI+] repressed Sup35 function as a translation terminator and allowed read-through of stop codons. Generation of both [URE] and [PSI+] seems to be last resort tactics for stressed yeast to survive poor environmental conditions without resorting to genetic mutation. Progeny yeast inherit both elements as non-Mendelian dominant traits, a phenomenon that baffled yeast geneticists for decades (Cox 1994). But incredibly astute observations and elegant experimentation by Wickner not only neatly solved the mystery of these heritable elements, but also lent the prion hypothesis much-needed independent corroboration as a biological paradigm in a completely different eukaryotic model system. Ure2p and Sup35 are absolutely required to generate and maintain [URE3] and [PSI+], both of which appear more frequently when Ure2p and Sup35 are overexpressed. Growth in the presence of 5 mM guanidine hydrochloride cured yeast of either prion. Like the proposed mammalian prions, yeast prions were shown to template the misfolding of their normal protein isoforms to prions (Glover et al. 1997). Sue Liebman soon found yeast prions to be dependent on heat shock protein 104 (Hsp104) for propagation (Chernoff et al. 1995), which launched investigations into the structural biochemistry of yeast prions. The ease and rapidity of generating yeast prions greatly accelerated studies investigating their structure and propagation, the data from which informed studies into the biochemical and structural bases for mammalian prion strains (Liebman 2001; Cascarina and Ross 2014).

PRION STRUCTURE ENCODES PRION STRAINS—CONFORMATIONALLY ENCODED EPIGENOMIC INFORMATION TRANSFER

Before the advent of the prion hypothesis, many researchers investigated apparent strain properties of the causative agent of TSEs. Cuillé and Chelle, then Pattinson decades later, described different clinical signs and incubation times in sheep and goats inoculated with the same sheep scrapie preparation (Cuille and Chelle 1938; Pattison and Jones 1967). Laboratory rodent bioassays greatly accelerated studies into TSE agent properties (Chandler 1961, 1963) and revealed species barriers to TSE material isolated from various animals that evoked descriptions of TSE agent strains (Fraser and Dickinson 1968; Bruce, Dickinson and Fraser 1976). While most assumed at this point the causative agent to be a virus (Alper, Griffith and Pattinson excluded), no viral preparation had thus far been isolated to directly analyze agent strain properties. So researchers relied on neuropathologic lesion profiling and incubation times within the host to characterize TSE strains.

At the same time Sanger revolutionized pathogen phylogenetic analyses with his chain termination method of sequencing DNA (Sanger, Nicklen and Coulson 1977), the TSE agent remained elusive. While DNA sequencing revealed genetic relationships among many conventional pathogens, it revealed only that the TSE agent, by now championed by Prusiner as a prion, was apparently encoded by a host gene that the pathogen itself did not express. While this seeming paradox bolstered the prion hypothesis, it also begged the questions: How do prion strains exist, and how does one define them, without genetic information? Looking beyond clinical disease course and pathology in the host to define prion strains, biochemists began investigating structural changes of PrP^{C} into the misfolded, pathologic form, PrP^{Sc}. In 1995, Richard Bessen and Byron Caughey identified non-genetic propagation of two distinct prion strains (Bessen et al. 1995), reminiscent of the yeast prions discovered a year earlier. Because two structurally distinct prions emerged from identical amino acid sequences, Caughey reasoned that these differences must be structurally determined. And so prion biochemistry became the Rosetta Stone with which to decipher the prion strain code.

Mammalian PrP^{C} ranges in size between 30 and 35 Kd depending on the status of glycosylation at two Asparagine residues near the C-terminus. Solution nuclear magnetic resonance (NMR) structural analyses and circular dichroism (CD) spectroscopic studies revealed a well-ordered C-terminal half of PrP^{C} containing three alpha helices comprising approximately 42% of its defined structure, with two small beta sheets contributing 3% (Riek et al. 1996; Hornemann et al. 1997; Liu et al. 1999). A disulfide bridge connects α-helices two and three. The remaining N-terminal half of PrP^{C} is disordered, adopts no consistent structure and has yet to be crystalized.

PrP^{Sc}, the misfolded form that correlates with infectivity, derives from PrP^{C} but exhibits starkly different physicochemical properties. PrP^{Sc} precipitates as an insoluble, detergent and protease-resistant aggregate of a core 27–30 Kd protein fragment (PrP 27–30) that retains infectivity (Pan et al. 1993). CD spectroscopy revealed a conformational transition from α-helices to predominantly β-sheets (54%) that differentiates PrP^{Sc} from PrP^{C}. Proteinase K accessibility, glycoform ratios and conformational stability in the presence of powerful chaotropes reveal structural, biochemical and stability differences among different prion strains. These biochemical and structural differences augment the biological and pathological characterization of prion strains (Safar et al. 1993, 1998; Bessen and Marsh 1994; Telling et al. 1996). Researchers discovered that these traits were heritable and used these criteria to characterize interspecies and intraspecies prion strains for scrapie, BSE, CJD, TME and CWD. Amazingly, unique prion strains maintain their unique biochemical signatures, as well as clinical and neuropathological signs, upon transmission to new individuals. Since no genetic instructions transmit with the prion to new hosts, prion strain structure must encipher and propagate prion strain signatures. The prion hypothesis therefore asserts a new paradigm of information storage and transfer in biological systems.

Comparison of PrP^{C} primary amino acid sequences, secondary structures and relative conversion and transmission efficiencies reveal interesting insights and possible biochemical

explanations for host ranges, species barriers and prion strains. Seminal transgenetic studies demonstrated that homology between host PrP^C and PrP^{Sc} primary sequences primarily dictates prion strain replication competency (Prusiner et al. 1990). Primary sequence differences can translate into secondary and tertiary structural differences that can profoundly impact strain susceptibility of the host. For example, the L1 loop connecting β-sheet one to α-helix two can be variably flexible among different animal species and affect propagation of different strains both in vivo and in vitro (Gossert et al. 2005; Gorfe and Caflisch 2007; Kurt et al. 2009, 2014; Striebel et al. 2011; Kyle et al. 2013; Angers et al. 2014). This genetically encoded conformational polymorphism, as well as others of the nearly 30 PrP polymorphisms (Prusiner and Scott 1997), may help control another important aspect of prion strain replication—nucleation-dependent polymerization (Come, Fraser and Lansbury 1993).

Prion replication occurs much more rapidly when a PrP^{Sc} nucleus, or 'seed' templates misfolding of PrP^C (Aguzzi and Weissmann 1997). But how does this seed or nucleus form? PrP^C likely faces a significant thermodynamic barrier to misfold into PrP^{Sc}. In genetic prion diseases like sporadic CJD, GSS or fatal familial insomnia, PrP^C harbors polymorphisms which may lower this energy barrier, and occasionally PrP^C converts to PrP^{Sc} at a very slow, limiting rate. Reverting back to PrP^C may require even more energy after misfolding, effectively locking PrP^{Sc} in that conformation. Primary PrP^C sequence largely dictates the range of PrP^{Sc} conformations with which it can interact. PrP^{Sc} molecules may now coerce other PrP^C molecules to misfold, perhaps by lowering the PrP^C to PrP^{Sc} energy barrier even further, as proposed in the heterodimer model. Once established, this seed now stabilizes other PrP^{Sc} molecules in that conformation, greatly accelerating the process. PrP^{Sc} oligomers assemble into higher order fibrils, which clump into larger, amyloid aggregates that deposit in the brains of affected animals as detergent, protease and acid resistant plaques characteristic of TSEs.

EXPLORING PRION FRONTIERS

Researchers are now expanding the prion paradigm to include other normal host proteins that misfold to cause diseases (Miller 2009; Prusiner 2012; Walker and Jucker 2015), including Alzheimer's, Parkinson's and Huntington's diseases (Ren et al. 2009; Polymenidou and Cleveland 2012), Amyotrophoic Lateral Sclerosis (Ludolph and Brettschneider 2015), Serum A Amyloidosis (SAA) (Murakami, Ishiguro and Higuchi 2014) and even Type 2 diabetes (Epstein et al. 2000; Khemtémourian et al. 2008) and cancer (Forget, Tremblay and Roucou 2013). Whether these misfolded proteins are bona fide prions or the pathologies they cause can truly be categorized as prion disorders remains a hotly debated issue. Prusiner originally defined prions in the context of infectious disease, as reflected in the acronym 'prion', which refers to a proteinaceous infectious particle (Prusiner 1982). Calling diseases like Alzheimer's and Parkinson's diseases infectious, or even transmissible, may be a bridge too far to cross. Classic prion diseases like scrapie, BSE and CWD most certainly transmit classical prions, containing aggregated PrP^{Sc}. But certainly mounting evidence demonstrates intraorganismal, if not interorganismal, transmission of these non-classical prions, which some have denoted 'prionoids' (Ashe and Aguzzi 2013; Liberski 2014). Aggregated $A\beta$ experimentally inoculated into mice seeded de novo generation of $A\beta$ and transmitted disease (Kane et al. 2000; Morales et al. 2012; Stöhr et al. 2012). So too did Lewy bodies and pathological α-synuclein transmit to normal brains to cause Parkinson-like diseases (Li et al. 2008; Luk et al. 2012; Watts et al. 2013). Experimental transmission of tauopathies via injection of mutant, pathologic tau protein to mice expressing normal tau also supports the transmissibility of non-classical prions. Clearly, these other misfolded proteins behave biochemically as prions, prompting Lary Walker and Mathias Jucker to propose a new definition of prions as 'proteinaceous nucleating particles' (Walker and Jucker 2015). One could certainly argue that genetic prion diseases are not really infectious, but rather transmissible, as has been demonstrated iatrogenically (Rappaport 1987). Kuru is likely just sporadic CJD transmitted by the abhorrent practice of cannibalism, not natural infection. Experimental evidence suggests that diseases like AD and PD can also be transmitted, but are not naturally infectious either. Perhaps the concept of prions could encompass classical prions that fulfill both the infectious and biochemical components, as do TSEs and even SAA (Murakami, Ishiguro and Higuchi 2014); and non-classical prions that fulfill the biochemical component of nucleation, propagation and transmission of amyloid.

If misfolding of a host protein can cause pathology, they might also control certain normal physiological responses too, as is the case with the yeast proteins Ure2p and Sup35? Could protein shape-shifting be an inherent biochemical property contributing to normal physiological functions in higher eukaryotes? Recently, Hou and Chen described a prion-like mechanism in the mammalian innate immune system. The RIG-I-mediated antiviral response induces mitochondrial antiviral signaling (MAVS) proteins to alter their conformation to assemble into prion-like aggregates that transduce signals for type I interferon production (Hou et al. 2011). MAVS aggregates can also seed monomeric MAVS to refold into detergent and protease-resistant aggregates. Like the pathologic non-classical prions, MAVS certainly are not infectious. But they do fulfill the biochemical definition of prions. Is that enough to call MAVS prions? That depends on expanding the prion concept to include those proteins that act only biochemically as prions. Without falling into a semantic argument, perhaps the focus should be on an emerging paradigm in biology that reaches beyond infectious disease. While MAVS represents the first mammalian exemplar of functional prions discovered thus far, researchers have found several physiologically functional prions in yeast. So rather than being a rare, pathological phenomenon that breaks infectious biological dogma, prions may reveal physicochemical properties inherent in proteins that can dictate physiological as well as pathological processes. Scientists eagerly anticipate discovering new prions with diverse new properties and roles in the research frontier that lies ahead.

Conflict of interest. None declared.

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