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Prion strains: shining new light on old concepts

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Abstract

Prion diseases are a group of inevitably fatal neurodegenerative disorders affecting numerous mammalian species, including humans. The existence of heritable phenotypes of disease in the natural host suggested that prions exist as distinct strains. Transmission of sheep scrapie to rodent models accelerated prion research, resulting in the isolation and characterization of numerous strains with distinct characteristics. These strains are grouped into categories based on the incubation period of disease in different strains of mice and also by how stable the strain properties were upon serial passage. These classical studies defined the host and agent parameters that affected strain properties, and, prior to the advent of the prion hypothesis, strain properties were hypothesized to be the result of mutations in a nucleic acid genome of a conventional pathogen. The development of the prion hypothesis challenged the paradigm of infectious agents, and, initially, the existence of strains was difficult to reconcile with a protein-only agent. In the decades since, much evidence has revealed how a protein-only infectious agent can perform complex biological functions. The prevailing hypothesis is that strain-specific conformations of PrP^{Sc} encode prion strain diversity. This hypothesis can provide a mechanism to explain the observed strain-specific differences in incubation period of disease, biochemical properties of PrP^{Sc}, tissue tropism, and subcellular patterns of pathology. This hypothesis also explains how prion strains mutate, evolve, and adapt to new species. These concepts are applicable to prion-like diseases such as Parkinson's and Alzheimer's disease, where evidence of strain diversity is beginning to emerge.

Keywords

Prion strain diversity; SSBP/1; Synthetic prions; Biological stability

Introduction

Prion diseases are transmissible neurodegenerative disorders that affect mammals and are inevitably fatal. Prion diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and other cervids, and Creutzfeldt-

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Jakob disease (CJD) in humans. Prions are comprised solely of PrP^{Sc}, the self-templating disease-specific conformation of the host cellular prion protein, PrP^{C} (Bolton et al. 1982; Caughey and Raymond 1991; Deleault et al. 2007; Oesch et al. 1985; Prusiner 1982; Wang et al. 2010). Prion disease is characterized by a long subclinical incubation period followed by a comparatively short clinical phase of disease and spongiform degeneration and accumulation of PrP^{Sc} in the central nervous system (CNS) (Comoy et al. 2015; Jeffrey et al. 1992; Masters et al. 1984; Swerdlow et al. 2003).

Prions exist as strains, which are operationally defined as a heritable phenotype of disease under defined conditions. These conditions include the titer of the agent, the route of inoculation, and the PrP amino acid sequence of both the agent and host. The resultant strain-specific phenotype of disease can be classified by differences in incubation period and neuropathology (e.g., lesion profile, PrP deposition patterns), but clinical signs and biochemical features of PrP^{Sc} can also help differentiate strains. Lacking nucleic acid, strain properties are hypothesized to be encoded by strain-specific conformations of PrP^{Sc} and are variations in the misfolding of PrP^{Sc} (Bartz 2016; Bessen and Marsh 1994; Kascsak et al. 1987; Telling et al. 1996). Consistent with this hypothesis, strain-specific differences in the biochemical features of PrP^{Sc} correspond with different prion strains. However, the relationship between the conformation of PrP^{Sc} and the phenotype of disease is poorly understood.

Prion strain diversity was first identified in the 1960s. In the 1970s, transmission of prions to rodents resulted in the identification and characterization of numerous prion strains and identification of many of the host and agent factors that influence strain diversity. More recent studies have started to unravel the relationship between strain-specific conformations of PrP^{Sc} and strain properties. Here we review the historical development of prion stain studies, recent findings examining the nature of how a protein-only infectious agent can cause strain diversity, and how these new findings provide insight into historical studies of prion strains.

Classical strains

Brief history of prion strains

Scrapie, the first described prion disease, was determined to be transmissible following experimental inoculation of brain homogenate from a scrapie-infected sheep to a healthy sheep (Cuillé and Chelle 1936; 1939). In 1950, an isolate of scrapie, Sheep Scrapie Brain Pool 1 (SSBP/1), was established following nine intracranial (i.c.) serial passages in sheep without noticeable clinical or neuropathological changes (Wilson et al. 1950). The pool was from three natural cases of scrapie (one Cheviot and two Cheviot × Border Leicester crosses) and was passaged mainly through Cheviot sheep (Dickinson 1976). The attack rate of scrapie in sheep was low, making investigations of scrapie pathogenesis and biochemistry difficult. To accomplish these studies, attention turned to i.c. transmission of scrapie to goats (Fig. 1), in which natural scrapie is known to occur (Chelle 1942). Transmission of SSBP/1 to goats resulted in a 100% attack rate and the clinical and neuropathological characteristics of scrapie infection in goats were congruent with the SSBP/1 isolate in sheep (Pattison 1957). Interestingly, serial passage of SSBP/1 in goats led to the emergence of two distinct

clinical phenotypes, “scratching” and “drowsy,” indicating that there may be different strains of the infectious agent (Pattison and Millson 1961). Genetic diversity in goats as the cause of these distinct phenotypes was excluded, and it was hypothesized that co-infection with a conventional pathogen, such as bacteria or a virus, was modifying the infectious agent. This possibility was eliminated, as strain characteristics remained unchanged following treatment of the scrapie agent with boiling, formalin, or biological cloning (Dickinson 1976). Since these distinct disease phenotypes remained unchanged following repeated passage, the differences observed between “scratching” and “drowsy” goats was concluded to be due to agent-strain differences.

Strain characterization studies in goats were hindered by long, variable incubation periods (Dickinson 1976). Successful transmission of scrapie from sheep and goats to rodents (e.g., mice) overcame this challenge, allowing for further characterization of prion strains (Fig. 1). Murine-adapted scrapie recapitulated the clinical and neuropathological features of natural prion disease but with shorter incubation periods and higher attack rates compared to goats. Additionally, a larger number of mice could be infected compared to goats, allowing for more robust study design. Passage of the SSBP/1 brain pool, Suffolk sheep scrapie, and both “scratching” and “drowsy” goat isolates to different lines of inbred mice led to the emergence and identification of a variety of prion strains (Figs. 1 and 2) (Bruce 1993; Bruce and Fraser 1991; Bruce et al. 1991; Dickinson 1976; Dickinson and Meikle 1971; Zlotnik and Rennie 1962, 1963). The strains of scrapie in mice had distinct clinical phenotypes of disease, incubation periods, and neuropathology (e.g., lesion profile), characteristics used to classify prions strains to this day (Dickinson 1976; Dickinson et al. 1968; Fraser and Dickinson 1968).

Transmission of different strains of murine-adapted scrapie to hamsters resulted in the isolation of hamster-adapted murine strains (Fig. 1). Compared to mice, hamster prion strains can have shorter incubation periods and higher infectivity titers in the brain (Kimberlin and Walker 1977; Marsh and Kimberlin 1975). The inverse relationship between infectivity titer and incubation period of disease was first observed in hamsters (Marsh and Kimberlin 1975). More recently, transmission of prion isolates to bank voles has resulted in distinct bank vole–adapted strains that have shorter incubation periods compared to hamsters (Di Bari et al. 2013; Nonno et al. 2020). Bank voles also are susceptible to prions from a greater range of species and strains compared to either mice or hamsters (Agrimi et al. 2008; Cartoni et al. 2007, 2005; Di Bari et al. 2008, 2013; Nonno et al. 2006, 2020; Piening et al. 2006; Pirisinu et al. 2016; Watts et al. 2014b; Zanusso et al. 2007). Overall, transmission of scrapie to rodents greatly aided the ability to study prion disease in vivo and serial passage of strains through these species provided stable, highly reproducible strains for studying prion strain concepts. With all rodent-adapted prions, these are not prion models but instead are bona fide prion-infected animals that faithfully recapitulate prion replication, pathogenesis, and strain properties (Chandler 1961; Dickinson 1976; Fraser and Dickinson 1968; Kimberlin and Marsh 1975; Kimberlin and Walker 1986, 1989; Watts et al. 2014b). This is in stark contrast to transgenic (Tg) mice used in other protein misfolding diseases of the CNS (e.g., Alzheimer’s, Parkinson’s) that recapitulate some, but not all, of the properties of disease.

The SSBP/1 transmission line

Transmission of SSBP/1 to rodents was a significant event in prion research. The strains derived from SSBP/1 are the basis of much of the research in the prion field. Strains derived from the SSBP/1 isolate can be divided into two “groups”: the SSBP/1 group and the “drowsy goat” group (Kimberlin et al. 1989). The SSBP/1 group consists of strains isolated from direct transmission of SSPB/1 to mice (i.e., 22L, 22C, 22A) and later hamsters (i.e., 22LH, 22CH, 22AH) (Fig. 1). The “drowsy goat” group consists of strains isolated from passage of SSBP/1 to goats, mice (i.e., 79A, 79 V, 139A/Chandler, RML), and hamsters (i.e., 79A, 79 V, 139H) (Fig. 1).

Multiple rodent-adapted prion strains are associated with the 139A/Chandler passage line (Fig. 1). An isolate of 139A/Chandler from a Swiss mouse was sent to Rocky Mountain Laboratories (RML) where 139A continued to be passaged in a closed colony of CD-1 Swiss mice where it was renamed RML (Prusiner et al. 1990). After three passages of “drowsy goat” scrapie in mice, brain material was transmitted to rats prior to transmission to hamsters (Chandler and Fisher 1963). After one passage in hamsters, brain material was sent to researchers in Compton, UK and Madison, WI for a second passage in hamsters (Fig. 1). Following serial passage in hamsters at Compton, Kimberlin and Walker isolated a short-incubation period, hamster-adapted strain termed 263 K (Kimberlin and Walker 1977, 1978). Researchers at Madison also serially passaged this brain material in hamsters, sending material from the sixth serial passage to the University of California at San Francisco where it was passaged four more times in hamsters and designated the hamster-adapted strain Sc237 (Fig. 1) (Kimberlin and Marsh 1975; Marsh and Kimberlin 1975; Prusiner et al. 1980; Scott et al. 1989). Since Sc237 shares clinical properties with 263 K and has a similar passage history, it is almost certain that Sc237 and 263 K are the same prion strain (Scott et al. 1989).

In addition to SSBP/1, natural scrapie from sheep (e.g., Suffolk scrapie spleen pool) was transmitted to mice numerous times, often resulting in isolation of the same murine-adapted strain, ME7 (Fig. 2) (Dickinson 1976; Zlotnik and Rennie 1963, 1965). Analysis of transmission of natural sheep scrapie to mice from 26 sources around the UK found that over half the mice developed prion disease consistent with infection with ME7 (Dickinson 1976). Similar to the strains derived from SSBP/1, ME7 is able to transmit to hamsters (ME7H; Fig. 2) (Kimberlin et al. 1989). Overall, transmission studies in rodents revealed that prions, like more conventional pathogens, exist as strains and a single host species can support several diverse strains.

Prion strain characteristics

Strains can be distinguished by differences in clinical signs, the incubation period of disease, and, most importantly, neuropathology. Strain-specific clinical signs were first observed when scrapie was transmitted to goats and “scratching” and “drowsy” clinical syndromes emerged (Pattison and Millson 1961). Transmission of transmissible mink encephalopathy (TME) to hamsters led to the emergence of two distinct hamster-adapted strains, one characterized by clinical signs of hyperexcitability and ataxia (Hyper–HY) and the other characterized by clinical signs of progressive lethargy (Drowsy–DY) (Bessen and Marsh

1992a, b). Certain prion strains can have similar clinical signs of disease. Obesity is observed with murine strains 22L and ME7 (in SJL mice) and hamster strains 139H and 22CH (Carp et al. 1984, 1990). Hyperesthesia and incoordination are observed with hamster strains 263 K, HY and HaCWD (Bartz et al. 1998; Bessen and Marsh 1992a; Kimberlin and Walker 1977). Therefore, clinical signs alone cannot be used to distinguish strains.

The incubation period of prion disease is remarkably consistent and reproducible under controlled experimental conditions (Bruce et al. 1991; Dickinson and Outram 1979; Westaway et al. 1987). These conditions include the titer of the inoculum, the route of inoculation, and PrP genotype. The titer of the prion agent used in transmission studies has a large effect on the incubation period of disease; however, when titer is controlled for, prion strains can have vastly different incubation periods. In mice the incubation periods can range from approximately 150 to 500 days post infection (Outram 1976). The route of inoculation can also dramatically affect the incubation period of disease. Direct inoculation of the central nervous system results in shorter incubation periods compared to extraneural routes of infection such as per os or intraperitoneal inoculation. The vast majority of strains behave similarly regarding the route of infection and the titer of agent; however, polymorphisms in the prion protein gene (*Prnp*) can result in strain-specific changes in the outcome of disease. Prior to discovery of the prion protein gene, control of the incubation period of prion disease in sheep and mice was linked to the genes *Sip* and *Sinc*, respectively. Once the protein-only hypothesis of scrapie was proposed and the prion protein gene identified, the *sinc*, *sip*, and *Prnp* genes were found to be congruent, and the *sinc* and *sip* genes were redesignated as *Prnp* (Carlson et al. 1986; Carp et al. 1987; Hunter et al. 1987; Moore et al. 1998; Westaway et al. 1987). For historical accuracy, the *Prnp* gene in mice will be referred to by its original designation, *sinc*. In mice, the *Sinc* gene has two alleles: *s7*, which ***s***hortens the incubation period of strain ME7 in mice, and *p7*, which ***p***rolongs the incubation period of ME7 in mice (Dickinson et al. 1968). This gene was found to affect the incubation periods of other murine strains that could be divided into two groups (the ME7 group and 22A group) based on the length of incubation period in $Sinc^{s7/s7}$ versus $Sinc^{p7/p7}$ homozygous mice (Dickinson and Outram 1979). The ME7 group consisted of strains with shorter incubation periods in $Sinc^{s7/s7}$ compared to $Sinc^{p7/p7}$ homozygous mice (e.g., ME7, 79A, 79 V, 139A) and the 22A group consisted of strains with shorter incubation periods in $Sinc^{p7/p7}$ compared to $Sinc^{s7/s7}$ homozygous mice (e.g., 22A, 22F, 87 V). Interestingly, following passage of scrapie field isolates to mice, ME7 group strains were often isolated when passaged in $Sinc^{s7/s7}$ mice, and 22A group strains were often isolated when passaged in $Sinc^{p7/p7}$ mice, i.e., within the *Sinc* genotype where incubation periods were shortened (Dickinson and Outram 1979).

Transmission of most prion strains to S $inc^{s7/p7}$ heterozygous mice resulted in an incubation period intermediate between $Sinc^{s7/s7}$ versus $Sinc^{p7/p7}$ homozygous mice as predicted by Mendelian genetics. A subset of prion strains, however, had an incubation period in $Sinc^{s7/p7}$ heterozygous mice that was longer than either $Sinc^{p7/p7}$ or $Sinc^{s7/s7}$ homozygous mice. This overdominance suggested that the gene product of *Sinc* (PrP) acted as a dimer or multimer in prion pathogenesis (Dickinson and Outram 1979). Later investigations of overdominance in transgenic mice expressing ovine PrP supported and clarified these observations (Saijo et

al. 2013). Polymorphisms at residue 136 in sheep PrP affect susceptibility to scrapie, with valine (V/V-136) or alanine (A/A-136) increasing susceptibility or conferring resistance to scrapie infection, respectively. Transgenic mice heterozygous at residue (A/V-136) infected with SSBP/1 displayed overdominance, with a shorter incubation period than either V/V-136 or A/A-136 transgenic mice infected with SSBP/1 (Saijo et al. 2013). The clinical, biochemical, and pathological features of SSBP/1-infected transgenic A/V-136 mice were similar to SSBP/1-infected transgenic V/V-136 mice. Western blotting of SSBP/1-infected A/V-136 brain homogenate using the PRC5 antibody (preferentially recognizes A136 polymorphism), however, revealed substantial amounts of A136 PrP^{Sc}. Additionally, serial PMCA determined that coexpression of V136 PrP^{C} with A136 PrP^{C} in heterozygous transgenic mice, A136 PrP^{C}, can be converted by SSBP/1, whereas A-136 PrP^{C} in homozygous A/A-136 transgenic mice is resistant (Saijo et al. 2013). These results suggest that V136 PrP^{Sc} can cross template A136 PrP^{C} to PrP^{Sc}, conferring strain properties indistinguishable from V136 PrP^{Sc}. Thus, this study supports historical data that overdominance occurs when a dominant prion conformer directly interacts with a resistant PrP gene product, rendering it susceptible to conversion. Overall, polymorphisms in murine PrP affect the length of the incubation period and, likely, affect prion strain emergence in mice.

Not all prion strains have distinct incubation periods. The murine strains 79A and 139A have incubation periods in *Prnp*^{a} mice around 200 dpi, and the short-incubation period hamster strains 263 K and HY have incubation periods around 60–70 dpi (Bessen and Marsh 1992a; Kimberlin and Walker 1977). Since determining the incubation period of disease relies on identification of clinical signs, which can range from subtle to prominent, a less subjective method of strain identification based on neuropathology was developed.

Characterization of neuropathology, specifically the location and severity of spongiosis and PrP^{Sc} deposition in the CNS, can aid in prion strain identification. To facilitate neuropathological comparisons among strains, Fraser and Dickinson devised a lesion scoring system evaluating nine anatomical brain regions for severity of spongiosis, assigning each region a score of 0 (no vacuoles) to 5 (confluent vacuoles) (Fraser and Dickinson 1968). The output of this system was termed a “lesion profile,” and this method is still widely utilized to compare the neuropathology of strains. The pattern of PrP^{Sc} deposition within the CNS also corresponds with prion strains. Histoblot analysis provides low anatomical resolution deposition patterns of PrP^{Sc} in rodent CNS tissue. This technique has shown that the regional distribution of PrP^{Sc} can greatly vary between prion strains and has the advantage of being able to rapidly identify gross changes in PrP^{Sc} deposition patterns (Ayers et al. 2009; Brandner et al. 2008; Bruce et al. 1989; DeArmond et al. 1993; Gonzalez et al. 2002, 2003; Jeffrey et al. 2006, 2014; Martin et al. 2009; Schoch et al. 2006; Ye et al. 1998). Overall, the neuropathology, incubation period, and clinical signs of prion disease are the principal features of prion disease utilized to classify strains.

Biological stability of prion strains

Transmission of a strain to a host with a different PrP amino acid sequence can alter the properties of the strain. The extent of the change in strain properties is referred to

as biological stability (Bruce and Dickinson 1979). Murine prion strains segregate into three stability classes (Table 1). Class I (e.g., ME7) prion strains encompass strains that retain their identity irrespective of host PrP genotype or species. For example, transmission of ME7 from a *Sinc*$^{s7/s7}$ genotype to a *Sinc*$^{p7/p7}$ genotype results in an extension of the incubation period and alteration in the lesion profile compared to ME7 passaged in *Sinc*$^{s7/s7}$ mice. Serial transmission of ME7 in *Sinc*$^{p7/p7}$ does not result in a shortening of the incubation period or a change in the lesion profile. Back passage of ME7 from *Sinc*$^{p7/p7}$ to *Sinc*$^{s7/s7}$ mice results in an incubation period and lesion profile identical to the original ME7 in *Sinc*$^{s7/s7}$ mice. Consistent with the murine transmission data, passage of ME7 into sheep and then back to mice did not alter the strain properties of ME7 (Dickinson 1976). These data indicate that ME7 is not altered by passage in a different *Sinc* genotype. Class I strains illustrate an important property of the operational definition of prion strains in that the same strain (i.e., the same agent encoded information) has a different phenotype when host parameters are altered (e.g., PrP genotype, route of infection).

In contrast to class I strains, class II strains are stable when passaged in the same *Sinc* genotype but are altered when passaged in a different *Sinc* genotype (Table 1). For example, transmission of SSBP/1 scrapie to VM (*Sinc*$^{p7/p7}$) mice resulted in the isolation of murine-adapted scrapie strain 22A. When passaged within VM mice, 22A is stable, but passage to C57BL (*Sinc*$^{s7/s7}$) mice leads to a gradual shift in incubation period with a corresponding change in the lesion profile that stabilizes after four to five passages. The stabilized strain, 22F, is thought to be a mutant strain generated upon transmission to the new *Sinc* genotype since (i) the 22F strain is not observed in other passages of SSBP/1 to mice and (ii) the emergence of 22F occurs from the transmission of either uncloned 22A or biologically cloned 22A. This gradual shift and stabilization of prion strain properties upon serial passage in a host with a different PrP amino acid sequence is also observed during interspecies transmission and adaptation. This process can also produce mutants upon transmission to a host with a different PrP amino acid sequence, and the process of adaptation is the result of selection of a dominant strain from a mixture. Importantly, unlike class I strains, prion strains that have adapted to a new PrP amino acid sequence have reduced pathogenicity for the original PrP amino acid sequence requiring several passages for the strain to adapt. For reasons that are unknown, the re-adapted strain may or may not have the same strain properties as the original starting strain. Overall, class II strains require multiple passages to adapt to a new host PrP amino acid sequence (either within or between host species) that can lead to generation of mutant strains.

Class III strains are unstable, even within the *Sinc* genotype in which they were isolated (Table 1). For example, 87A was isolated following passage of SSBP/1 to C57BL *Sinc*$^{s7/s7}$ mice and is characterized by high incidence of amyloid plaques. Unlike other strains, passage of 87A can result in an abrupt shortening of the incubation period and emergence of a new strain in one passage, termed 7D. This differs from the gradual change observed for class II strains. It was observed that the emergence of 7D from 87A correlated with the focal asymmetrical vacuolation in animals infected with 87A prior to the rapid emergence of 7D. Additionally, the emergence of 7D was observed more frequently when passages were performed at a high titer of 87A compared to a low titer. The newly emergent strain 7D has an incubation period and lesion profile that is indistinguishable from the murine-adapted

strain ME7. Importantly, the rapid, single passage, emergence of 7D from 87A was observed from biologically cloned 87A and also when 87A was passaged at a high dilution of brain homogenate where 7D failed to cause disease by the i.c. route of infection. These data suggest that 7D was not present in the 87A inoculum but instead arose during passage of 87A in mice. Also of interest is that several independent transmissions of SSBP/1 to mice identified strains that were indistinguishable from 87A (31A, 51C, 125A, 138A), and these strains also exhibited class III strain behavior and always broke down to a strain resembling 7D. Furthermore, the reverse of class III behavior was never observed, that is, 7D never changed to 87A or another strain. This was attributed to the loss of the informational molecule, presumably a nucleic acid; however, it was unclear why a conventional pathogen, with a nucleic acid genome, would always mutate to the same strain.

Modern strains

Development of synthetic prions—The development of in vitro methods to generate PrP^{Sc} de novo provided concrete evidence to support the protein-only hypothesis. The cell-free conversion assay used [^{35}S]-labeled PrP^{C} as substrate, leading to formation of radioactive nascent PrP^{Sc}, which co-aggregated with unlabeled PrP^{Sc} seed. The cell-free conversion assay confirmed the seeding capability of PrP^{Sc} as well as fidelity of strain-specific conformations (Bessen et al. 1995; Kocisko et al. 1994). Protein misfolding cyclic amplification (PMCA) modified this concept by adding multiple rounds of prion amplification (Saborio et al. 2001). In PMCA, a small amount of prion-infected brain homogenate is added to normal, uninfected brain homogenate. During the incubation phase, PrP^{C} binds to PrP^{Sc} and is converted into new PrP^{Sc}. The sonication step breaks PrP^{Sc} aggregates apart, creating more free ends for prion conversion. This process of incubation and sonication is repeated, leading to the exponential amplification of PrP^{Sc} and the subsequent depletion of PrP^{C}. To continue the amplification process, nascent PrP^{Sc} is added to fresh uninfected brain homogenate, for a new round of PMCA. This method allows for the indefinite propagation of PrP^{Sc} in vitro at much higher efficiency than the cell-free conversion assay (Saborio et al. 2001). Importantly, in vitro generated PrP^{Sc} is infectious in vivo and maintains the strain properties of the original PrP^{Sc} seed (Cali et al. 2019; Castilla et al. 2008a, b, 2005; Eckland et al. 2018; Green et al. 2008; Lucassen et al. 2003; Saa et al. 2012; Shikiya et al. 2010; Shikiya and Bartz 2011). PMCA is an important tool in prion research and plays an essential role in generation of de novo prions. The first attempts to develop synthetic prions used synthetic PrP peptides. Synthetic PrP peptides spanning amino acids 106 to 126 that contained a mutation associated with GSS were generated that formed amyloid fibrils, possessed high β-sheet content, were partially resistant to PK and pronase digestion, and triggered neuronal death in cell culture (De Giola et al. 1994; Forloni et al. 1993, 1996; Selvaggini et al. 1993; Tagliavini et al. 1993; Thellung et al. 2000). Similarly, a synthetic peptide corresponding to mouse PrP(89–143) with the P101L substitution (MoPrP[P101L]; murine equivalent of GSS P102L substitution) refolded into a β-sheet rich isoform. Inoculation of these synthetic peptides into Tg196 mice, which express MoPrP(P101L) at low levels and can spontaneously develop CNS dysfunction later in life, can increase the attack rate to 100%, shorten the incubation period, and cause neuropathological features of GSS (Kaneko et al. 2000). These studies established that synthetically derived PrP peptides could enhance development of prion disease in vivo.

Synthetic prions can be generated from full-length PrP. Based on a previous study investigating the formation of amyloid by the prion protein, mouse PrP spanning amino acids 89 through 230 (MoPrP[89–230]) was expressed in *E. coli*, purified, and formed into β-oligomers by incubation in urea and buffer (Baskakov et al. 2002). Two forms of amyloid fibrils were generated: "unseeded," formed by subjecting monomeric recMoPrP(89–230) at 37 °C to constant shaking, and "seeded," formed using preformed fibrils as seed (Legname et al. 2004). When i.c. is inoculated into transgenic mice (Tg9949) overexpressing MoPrP(89–230), both unseeded and seeded fibrils caused clinical signs of prion disease in all mice inoculated (Legname et al. 2004). Neuropathology differed between seeded- and unseeded-infected mice, suggesting that these synthetic prions are different strains (Legname et al. 2004). Synthetic prions from seeded-infected mice were termed mouse synthetic prion strain 1 (MoSP1; Table 2).

Synthetic prions can be generated from minimal components using PMCA. Synthetic prions were generated from unseeded (no PrP^{Sc}) substrates where only purified hamster PrP^{C} and poly(A) RNA were present (Table 2) (Deleault et al. 2007). Once formed, the de novo PrP^{Sc} could be serially propagated in PMCA and was infectious when inoculated into hamsters. Negative control reactions did not contain measurable infectivity in hamsters; however, to provide additional rigor to these findings, the experiment was replicated in a facility that had not been previously used for prion studies using new equipment and reagents. Importantly, de novo generation of PrP^{Sc} and prion infectivity was detected only in PMCA reactions containing PrP^{C} and RNA and was not detected in any of the subcomponents used for the study (Deleault et al. 2007). The hamsters that developed clinical signs of prion disease from the de novo generated prions had neuropathological hallmarks of prion infection, contained PrP^{Sc}, and prion infectivity, fulfilling Koch's postulate for an infectious agent (Deleault et al. 2007; Walker et al. 2006).

The use of PMCA accelerated the development of synthetic prions. With the successful generation of de novo prions using only PrP^{C} and RNA in PMCA (Deleault et al. 2007), other potential cofactors were investigated. While testing 16 different substrate and cofactor combinations, synthetic prions were generated de novo in serial PMCA by mixing bacterially generated recombinant murine PrP, RNA, and an endogenous lipid (1-palmitoyl-2-oleoylphosphatidylglycerol—POPG) (Wang et al. 2010). These murine synthetic prions (MSP) were highly infectious in mice, and confirmed minimal components in PMCA are sufficient to generate bona fide prions. Serial PMCA also produced the first de novo PrP^{Sc} molecule composed entirely from non-mammalian sources using bacterially generated recombinant murine PrP (recMoPrP) and a synthetic form of the lipid phosphatidylethanolamine (PE) as substrates (Deleault et al. 2012a). These synthetic prions were the first formed in the absence of nucleic acid cofactors, indicating that nucleic acids are not required for formation of infectious prions.

Generation of synthetic prions was not limited to PMCA (Table 2). Under denaturing conditions, recMoPrP was folded into a variety of amyloid fibrils by altering buffer conditions and temperature (Colby et al. 2009). Inoculation of these fibrils into transgenic mice resulted in the emergence of three strains with distinct clinical, biochemical, and neuropathological features. Testing various conditions also led to generation of protease-

sensitive synthetic prions which, when i.c. is inoculated into Tg mice, had an extended incubation period and caused extensive neurodegeneration (Colby et al. 2010). Purified recombinant hamster PrP could also be formed into fibrils under denaturing conditions followed by an annealing process in the presence of normal hamster brain homogenate (NBH) or bovine serum albumin (BSA) prior to inoculation (Makarava et al. 2010). Following i.c. inoculation into Syrian hamsters, neither the NBH- nor BSA-annealed fibrils caused clinical disease, but PK-resistant PrP was detected in the brains of 3/6 and 2/7 of inoculated animals, respectively. A second passage of NBH-annealed fibrils in hamsters resulted in the emergence of a long incubation period strain with highly distinct neuropathology characterized by large amyloid plaques (Synthetic Strain Leading to OverWeight—SSLOW). In contrast, clinical signs of prion disease were not observed until third passage with hamsters inoculated with BSA-annealed fibrils and only after an extended incubation period (Makarava et al. 2011). This stain, designated LOTSS (LOw Toxicity Synthetic Strain), was characterized by neuropathology consisting of large plaques, similar to SSLOW. The long adaptation process observed with both SSLOW and LOTSS led to development of the deformed templating model of prion conversion. The deformed templating model posits that the annealed synthetic fibrils do not consist of PrP^{Sc} but, through a trial- and-error process, are able to trigger formation of authentic PrP^{Sc} (Makarava et al. 2011, 2016, 2015). Overall, these studies indicated that a variety of methods can generate infectious synthetic prions (Table 2) and provided further support for the protein-only hypothesis.

Murine synthetic prion (OSU) transmission line—The murine synthetic prions generated by the method of Wang et al. in 2010 have furthered the understanding of the mechanisms of prion formation (Fig. 3) (Wang et al. 2010). The murine synthetic prion strain (termed the OSU strain) was used to examine the role of cellular host cofactors on prion strain properties and infectivity (Deleault et al. 2012b). Specifically, the OSU strain was propagated in PMCA for 30 rounds with only murine recombinant PrP and PE as substrate and cofactor, respectively. The resultant PMCA product was termed OSU cofactor PrP^{Sc}. OSU cofactor PrP^{Sc} was then subjected to an additional 18 rounds of PMCA where the cofactor PE was withdrawn, leaving only murine recPrP as substrate. Cofactor withdrawal resulted in PrP^{Sc} propagation with a change in the electrophoretic migration of the PK-resistant core. This propagated PrP^{Sc} was termed “protein-only PrP^{Sc}” or OSU protein-only PrP^{Sc} (Fig. 3) (Deleault et al. 2012b). Importantly, OSU protein-only PrP^{Sc} lacked in vivo infectivity and could not seed PrP^{Sc} formation in serial PMCA using murine brain homogenate (Deleault et al. 2012b). The results of this study established PrP alone is sufficient for prion propagation in vitro and suggest that cofactors play a critical role in prion infectivity.

To further investigate the role of cellular cofactors in prion infectivity, cofactor withdrawal experiments were repeated with OSU cofactor PrP^{Sc} (redesignated Mo cofactor recPrP ^{Sc}), this time using recombinant bank vole PrP (recBV PrP) as PMCA substrate (Burke et al. 2019). Bank voles, considered universal prion acceptors, were hypothesized to be more permissive to seeding by protein-only PrP^{Sc} compared to the original murine recPrP (Burke et al. 2019; Di Bari et al. 2008, 2013; Nonno et al. 2006; Watts et al. 2014b). Consistent

with the previous study, protein-only PrP^{Sc} formed and could be serially propagated in vitro with recBV PrP as sole substrate but lacked in vivo infectivity in bank voles (Fig. 3, BV M/I109 protein-only $recPrP^{Sc}$). In contrast, serial PMCA of Mo cofactor $recPrP^{Sc}$ with recBV PrP and PE was infectious in both bank voles and mice (Fig. 3, BV M109 cofactor $recPrP^{Sc}$). However, unlike the previous study with murine recPrP, recBV protein-only PrP^{Sc} could seed conversion of normal bank vole brain homogenate in PMCA (product termed [Protein-only → BH PrP^{Sc}]) and was infectious when inoculated into bank voles (Fig. 3, [Protein-only → BH PrP^{Sc}]). Titration of cofactor PrP^{Sc}, protein-only PrP^{Sc}, and [Protein-only → BH PrP^{Sc}] in bank voles found protein-only PrP^{Sc} was not infectious, but cofactor PrP^{Sc} and [Protein-only → BH PrP^{Sc}] contained a high prion titer. Therefore, infectivity of protein-only PrP^{Sc} could be restored upon exposure to cofactors in normal bank vole brain homogenate. The neuropathology of bank voles inoculated with either the original cofactor PrP^{Sc} or [Protein-only → BH PrP^{Sc}] was similar (Burke et al. 2019). When inoculated into bank voles, the neurotropism of cofactor PrP^{Sc} and [Protein-only → BH PrP^{Sc}] was the same, despite each prion using different substrates (recPrP versus brain-derived PrP) and cofactors (purified PE versus brain homogenate). This data led to the conclusion that protein-only PrP^{Sc} was structurally similar to cofactor PrP^{Sc}, faithfully transmitting this conformation to [Protein-only → BH PrP^{Sc}] (Burke et al. 2019; Supattapone 2020).

The results of this study have numerous implications. First, synthetic prions can overcome the species barrier in vitro. Second, the ability of protein-only PrP^{Sc} to replicate in vitro reinforces the previous findings that the prion protein alone is sufficient for self-templated replication. Third, similarities in strain properties between cofactor PrP^{Sc} and [Protein-only → BH PrP^{Sc}] suggest that the strain-specific conformation of cofactor PrP^{Sc} is maintained partially, or in full, in the absence of cofactors, suggesting that the prion protein alone retains strain identity. Fourth, the disparity in infectivity between protein-only PrP^{Sc} and [Protein-only → BH PrP^{Sc}] suggests that cofactor molecules are required for prion infectivity in animals; however, it is unclear what role cofactors play in conferring infectivity. One explanation offered by Burke et al. (2019) is that the global structure of PrP^{Sc} is maintained following cofactor withdrawal, but local conformational changes occur in the absence of cofactors that hinder in vivo amplification and can be reversed by reintroduction of cofactors in vitro (Burke et al. 2019). Overall, the role cofactors play in prion infectivity requires further study.

OSU synthetic prions (designated murine synthetic prions—MSP) can cross the species barrier in vivo (Block et al. 2021). Inoculation of Syrian hamsters with MSP resulted in the development of clinical signs of prion disease (Fig. 3). Interestingly, the strain that emerged following adaptation of the MSP to hamsters (HaMSP) was highly reminiscent of the brain-derived hamster strain 139H, which was isolated following transmission of 139A to Syrian hamsters (Fig. 1) (Block et al. 2021). This suggests that the interspecies transmission of the MSPs to hamsters recapitulated a known, specific interspecies transmission event, and the MSPs have 139A-like strain properties. Overall, the murine synthetic prions generated by Wang et al. (2010) have been the basis of several experiments that have greatly increased our understanding of prion biology.

Synthetic prions and strains—The environment used to generate synthetic prions can affect resultant disease phenotype. Fibrillization of purified hamster recombinant PrP under identical solvent conditions but differing agitation conditions—shaking (S) or rotating (R)—resulted in structurally distinct structures (Makarava and Baskakov 2008). Fibrils formed under shaking conditions (S fibrils) appeared curvy or S-shaped using electron microscopy or atomic force microscopy, whereas the fibrils formed under rotating conditions (R fibrils) had a linear rigid appearance. S and R fibrils can self-template their distinct conformations when used as seed for fibrillization reactions under opposing agitation conditions (i.e., S fibrils used as seed for fibrillization under rotating conditions and vice versa) indicating that the conformation of the fibril can self-template and is not entirely dependent on the reaction conditions (Makarava and Baskakov 2008). Generation of amyloids under conditions where the recMoPrP sequence, urea and salt concentrations, the buffer, pH, and temperature vary results in differences in conformational stability, fibril morphology, and infectivity in Tg4053 mice (Table 2) (Colby et al. 2009). Serial passage in Tg4053 mice led to isolation of distinct synthetic prion strains that differed in incubation period, biochemical properties of PrP^{Sc}, neuropathology, and ability to infect WT (FVB) mice (Colby et al. 2009).

Strains have been observed with non-PrP prions. For example, fibrillization of human recombinant A53T α-synuclein in the presence (S) or absence (NS) of salt generated distinct fibrils that differed in formation kinetics, ultrastructure, banding patterns following SDS-PAGE and Western Blot, and conformational stability. Importantly, inoculation of S or NS fibrils into T $gM83^{+/-}$ mice (overexpress A53T α-synuclein mutant) resulted in distinct disease phenotypes (Lau et al. 2020). Interestingly, the neuropathology caused by infection with the S fibrils was similar to the neuropathology observed when brain material from multiple systems atrophy patients is inoculated into $TgM83^{+/-}$ mice, while the neuropathology caused by infection with the NS fibrils was reminiscent of the neuropathology observed following inoculation of TgM83 mice with $M83^{+/+}$. These results indicate that the strain-specific properties of S and NS α-synuclein fibrils can recapitulate known strains of brain-derived synucleinopathies. Overall, synthetic prions generated with minimal components in a highly controlled environment provide a trackable system to study prion strains.

Prion strain typing methods—The biochemical properties of PrP^{Sc} can differentiate prion strains. The prion protein has two N-linked glycosylation sites which results in three potential glycosylation states: di-, mono-, or unglycosylated. Western blot analysis of the prion protein is characterized by a three-band pattern that corresponds to these three PrP glycoforms. Digestion of PrP^{Sc} with PK results in strain-specific susceptibilities and differences in the molecular weight of the PK-resistant core (Baron et al. 2000; Benestad et al. 2003; Bessen and Marsh 1992a, 1994; Kuczius and Groschup 1999; Parchi et al. 1996; Polak et al. 2008; Telling et al. 1996; Zou et al. 2010). Differences in the molecular weight of the PK-resistant core can be determined via Western blot, with PrP^{Sc} from different strains having different electrophoretic mobility. This has been observed in hamster, sheep, bovine, cervid, and human prion strains (Baron et al. 2000; Benestad et al. 2003; Bessen and Marsh 1992a, 1994; Casalone et al. 2004; Hope et al. 1999; Nonno et al. 2020; Parchi et al. 1999; Polak et al. 2008; Stack et al. 2002; Telling et al. 1996; Xie et al. 2006;

Zanusso et al. 2003). For example, the unglycosylated PrP polypeptide of PK digested PrP^{Sc} from either HY or type 1 sCJD migrates at 21 kDa while DY and Type 2 sCJD migrates at 19 kDa (Bessen and Marsh 1994; Zanusso et al. 2004). These differences in the migration of PK-digested PrP^{Sc} are believed to result from strain-specific conformations exposing different PK-cleavage sites (Bessen and Marsh 1994; Telling et al. 1996). Some anti-PrP antibodies can differentiate between the different PK resistant cores. For example, the monoclonal anti-PrP antibody 12B2 recognizes HY/Type 1 PrP^{Sc} but not DY/Type 2 (Langeveld et al. 2006). In addition to the migration of PrP^{Sc}, the relative ratio of the three PrP^{Sc} glycoforms can vary among strains and is used to categorize sporadic CJD (sCJD). Based on electrophoretic mobility and glycoform ratio, there are three distinct PrP^{Sc} patterns of sCJD and iCJD: Type 1, Type 2A, and Type U (Collinge et al. 1996; Parchi et al. 1999; Zanuss et al. 2007). Types 1 and 2A have similar glycoform ratios but migrate at 21 and 19 kDa, respectively. Type U is characterized by dominance of the unglycosylated form. A fourth pattern, type 2B, is found in vCJD and is characterized by migration at 19 kDa with a dominant diglycosylated glycoform (Collinge et al. 1996; Hill et al. 1997; Parchi et al. 1999; Will et al. 1996). Overall, the migration and relative ratio of the PrP^{Sc} glycoforms provide a powerful means to distinguish prion strains.

The relative resistance of PrP^{Sc} to denaturation differs between prion strains, consistent with the hypothesis that the conformation of PrP^{Sc} encodes strain diversity. The conformation-dependent immunoassay (CDI) quantifies the immunoreactivity of native and denatured PrP^{Sc}, plotting the ratio of bound denatured/native PrP against the concentration of PrP^{Sc} (Safar et al. 2000, 1998). The conformational stability assay (CSA) measures resistance of PrP^{Sc} to digestion with PK following denaturation with chaotropic agents such as sodium dodecyl sulfate (SDS) or guanidine hydrochloride (Gdn-HCl). The conformational stability and solubility assay (CSSA) is a conformational stability assay based on the differential solubility of PrP^{C} and PrP^{Sc}, using differential centrifugation followed by exposure to Gdn-HCl to measure PrP^{Sc} solubility as a function of increased exposure to Gdn-HCl (Pirisinu et al. 2010). These various PrP^{Sc} stability assays can differentiate both natural and synthetic prion strains (Ayers et al. 2011; Block et al. 2021; Colby et al. 2009; Deleault et al. 2012b; Ghaemmaghami et al. 2013; Legname et al. 2005, 2006; Peretz et al. 2001; Safar et al. 1998). In murine prion strains, a decrease in the conformational stability of PrP^{Sc} corresponds with a decrease in the incubation period of disease (Legname et al. 2006). Mechanistically, it is hypothesized that a decrease in PrP^{Sc} conformational stability increases fragmentation of PrP^{Sc}, thus creating more PrP^{Sc} free ends for prion formation to occur. Conversely, an increase in PrP^{Sc} conformational stability is hypothesized to decrease PrP^{Sc} fragmentation, leading to slower prion formation and a consequently longer incubation period. Consistent with this hypothesis, studies examining Sup35 yeast prions, tau, α-synuclein, and amyloid-β indicate that less stable fibrils have a higher propensity to undergo breakage, thereby producing new seeds for prion formation (Beal et al. 2020; Cohen et al. 2015; Kryndushkin et al. 2003; Lee et al. 2007; Ohhashi et al. 2018; Sang et al. 2018; Sun et al. 2008; Tanaka et al. 2004, 2006; Watts et al. 2014a; Xue et al. 2009; Zhou et al. 2009). Subsequent studies on hamster and human prion strains observed that this relationship is inverted, with PrP^{Sc} from short incubation period strains having higher conformational stability compared to strains with longer incubation periods (Ayers et al.

2011; Cescatti et al. 2016; Gonzalez-Montalban et al. 2011). Additionally confounding the observations made in mice and other species, these short incubation period strains (e.g., HY) with high PrP^{Sc} conformational stabilities are more efficient at PrP^{Sc} conversion in PMCA compared to long incubation period strains (e.g., DY) (Ayers et al. 2011). The mechanism responsible for the species-specific discrepancy in the relationship between PrP^{Sc} conformational stability and incubation period of disease is unknown. It is possible that the methods used to determine conformational stability of PrP^{Sc} do not accurately measure PrP^{Sc} fragmentation. It is hypothesized that the increased PrP^{Sc} conformational stability of short incubation period strains in hamsters may be a result of larger PrP^{Sc} aggregate sizes (Ayers et al. 2011); however, recent studies utilizing asymmetric-flow field-flow fractionation do not support this (Cortez et al. 2021). Overall, the structural basis of PrP^{Sc} conformational stability and its biological outcome are poorly understood.

The pattern of PrP^{Sc} accumulation, processing, and cellular response in the CNS can differentiate prion strains. The deposition pattern of PrP^{Sc}, as determined by immunohistochemistry, is strain-specific. This method, termed PrP^{Sc} (or PrP^{d}) profiling, assigns scores of 0 (absent) to 3 (severe) that detail the magnitude of PrP^{Sc} accumulation in six neuroanatomical sites (Jeffrey and Gonzalez 2007). In addition to abundance, the cellular processing of PrP^{Sc} is strain-specific. Immunohistochemistry using a panel of antibodies that recognize different epitopes along the length of the prion protein is termed “epitope mapping.” Studies in sheep using epitope mapping found strain-specific patterns of PrP^{Sc} truncation in neurons and glia (Gonzalez et al. 2002; Jeffrey and Gonzalez 2007; Jeffrey et al. 2003). In hamsters, epitope mapping observed differences in truncation patterns between short incubation period strains (i.e., HY, 263 K, HaCWD) and long incubation period strains (i.e., 22AH, 22CH, 139H, ME7H, DY) (Ayers et al. 2011). Full-length PrP was found in the neuropil of every strain examined, but N-terminally truncated PrP^{Sc} was only detected in the neuronal somata of short incubation period strains. Additionally, N-terminally truncated PrP^{Sc} was observed in astrocytes and microglia from every strain, but DY was slightly more truncated than the rest of the strains, but that is likely due to the difference in the PK cleavage site for DY PrP^{Sc} (Ayers et al. 2011). Finally, reactive astrogliosis is a prominent neuropathological feature of prion disease. Recently, IHC using an antibody against CD44, which is highly expressed in a subset of astrocytes in regions associated with prion disease, identified strain-specific patterns of CD44 upregulation in the hippocampus of between 15 distinct murine prion strains (Bradford et al. 2019). Overall, PrP^{Sc} deposition patterns, PrP^{Sc} process, and the glial response to prion-infection can be used to distinguish prion strains.

Biological stability revisited—Classical prion studies in mice identified that both the agent and the host contributed to the phenotype of the strain. The mechanisms underlying these observations were unknown, in large part due to the lack of understanding regarding the nature of the infectious agent. More recent work has not only identified that PrP^{Sc} is the infectious agent but has begun to identify mechanisms of how a protein-only infectious agent can perform complex biological functions such as the three classes of prion strains identified in mice.

Class I strains are stable irrespective of PrP genotype or host. For example, the incubation period and neuropathology of ME7 does not change between first and subsequent passages

in either *Sinc* genotype, lacking the adaptation period observed with class II strains. Additionally, ME7 is the strain most often isolated following transmission of scrapie to mice. Within a host species, certain strains are reisolated more often than others following interspecies transmission, which can lead to overrepresentation of these strains (Bruce and Dickinson 1979; Diaz-Espinoza and Soto 2010; Dickinson 1976; Huor et al. 2019). The frequent reisolation of ME7 suggests that the ME7 PrP^{Sc} conformation is favored. Importantly, ME7 does not have the shortest incubation period of known prion strains in either *Sinc*$^{s7/s7}$ or *Sinc*$^{p7/p7}$ mice. If the favored PrP^{Sc} conformation in mice correlated with prion conversion efficiency or incubation period, an intermediate incubation period strain like ME7 would not be expected to be isolated. Additionally, contamination of ME7 with shorter incubation period strains has never been detected. If a shorter incubation period strain were to contaminate ME7, the shorter incubation period strain would be expected to emerge as the dominant strain. This suggests that, in mice, thermodynamically favorable PrP^{Sc} conformations do not correlate with the shortest incubation period.

The characteristics of favored PrP^{Sc} conformations vary by host species. In hamsters, interspecies transmission of SSBP/1, the Stetsonville isolate of TME, and mule deer CWD resulted in the emergence of 263 K, HY TME, and HaCWD, respectively (Ayers et al. 2011; Bartz et al. 2000, 1998; Bessen and Marsh 1992a, 1992b; Kimberlin and Walker 1977, 1978, 1986; Kimberlin et al. 1989; Prusiner et al. 1980; Schutt and Bartz 2008; Scott et al. 1989). These three strains have similar incubation periods, PrP^{Sc} biochemical properties and neuropathologies (Ayers et al. 2011; Cortez et al. 2021; Schutt and Bartz 2008). The reisolation of the same strain in hamsters from sheep, mink, and mule deer from two continents spanning over 50 years was not only incompatible with a viral etiology but suggested that the conformation of hamster PrP^{Sc} that encodes these strains is favored. Additionally, it is hypothesized that the relatively high conversion efficiency and short incubation periods of these strains allow them to outcompete strains with relatively low conversion efficiency and long incubation periods. Prion evolution to a common strain has been observed with L and H type BSE evolving into C type BSE, murine synthetic prion MoSP1 evolving into an RML-like strain in FVB mice, and may occur with Norwegian CWD evolving into a strain that resembles North American CWD (Baron et al. 2011; Bian et al. 2021; Legname et al. 2005). Overall, reisolation of the same strain from diverse sources and evolution of prion strains to a common strain suggest that each PrP amino acid sequence possesses a finite number of thermodynamically stable conformations (i.e., strains), with the overrepresented conformations being the most thermodynamically favorable.

Class II prion strains require several serial passages in mice to stabilize the incubation period similar to what is observed upon interspecies transmission (Fig. 4). The deformed templating model offers an explanation of this observation that was originally developed to explain the transmission of hamster synthetic strain SSLOW to hamsters (Makarava et al. 2011, 2015). The deformed templating hypothesis posits that synthetic prions are not authentic PrP^{Sc} but instead consist of a fibrillar PrP conformation that, through an inefficient process of generating PrP^{Sc} folding intermediates, results in the production of atypical PK-resistant PrP prior to production of authentic PrP^{Sc} (Makarava et al. 2011, 2012). A similar process could occur in class II strains and interspecies transmission of brain-derived

prions. Mismatch in the amino acid sequence between PrP^{Sc} and host PrP^{C} may result in the inefficient generation of PrP^{Sc} and may lead to generation of conformational variants of PrP^{Sc} via deformed templating (Fig. 4). Since the conformation of PrP^{Sc} is hypothesized to encode prion strain diversity, changes in the conformation from the parental strain would be considered “mutations” as it is a heritable change in information. In other words, the conformation of PrP^{Sc} is an epigenetic means to encode information.

Serial transmission in the new host can result in the shortening and stabilization of the incubation period in class II strains and following interspecies transmission. The stabilization of the incubation period corresponds with the emergence of a dominant prion strain; the conformational selection model provides a mechanism for this process (Fig. 4). The conformational selection model proposes that within a population of newly produced PrP^{Sc} conformations, only a subset of the PrP^{Sc} will be able to convert the new host PrP^{C} to PrP^{Sc} (Collinge and Clarke 2007). The PrP^{Sc} conformations that can convert PrP^{C} of the new host to PrP^{C} will be selected for upon serial intraspecies transmission. Many factors are involved in the emergence of a dominant conformation of PrP^{Sc} (i.e., strain) from a mixture that include, but are not limited to, prion replication kinetics, prion strain cell and tissue tropism, and prion strain interference (Ayers et al. 2009, 2011; Bartz et al. 2004, 2007; Bett et al. 2012; Dickinson et al. 1972, 1975; Gonzalez-Montalban et al. 2013; Kimberlin and Walker 1985; Legname et al. 2006; Li et al. 2010; Mahal et al. 2010; Makarava et al. 2013; Mulcahy and Bessen 2004; Shikiya et al. 2010). Overall, when the agent PrP^{Sc} and host PrP^{C} amino acid sequences differ, within or between species, deformed templating can produce mutants, but the most fit strain(s) for that PrP amino acid sequence will emerge as the dominant strain.

Class III strains are unstable in the same PrP genotype. Unlike class II strains, changes in the strain properties of class III strains (1) occur within a singular PrP genotype (i.e., the genotype within which the strain is isolated) and (2) occur completely in a single passage versus several passages. Breakdown of 87A, or any other class III strain (i.e., 31A, 51C, 125A, 138A), always leads to shortening of the incubation period and a shift in lesion profile to a strain designated as 7D, a class I strain indistinguishable from ME7. The neuropathology of mice infected with 87A is characterized by high incidence of plaques, and the breakdown event is preceded by focal asymmetrical vacuolation. Breakdown to 7D/ME7 is accompanied by a shift in neuropathology to very low incidence of plaques and symmetrical vacuolation. Importantly, the breakdown of 87A was more frequently observed at high titer (i.e., low brain dilution) transmissions compared to passage at a lower titer (i.e., higher brain dilution). The breakdown of 7D to a strain with a longer incubation period and high plaque forming potential has not been observed. Based on this observation, the breakdown of 87A to 7D/ME7 was hypothesized to be irreversible due to a loss of genetic information when the scrapie agent was thought to contain a nucleic acid genome (Bruce and Dickinson 1979). Interpretation of these observations in light of PrP^{Sc} as the infectious agent would suggest that a given PrP sequence has a conformation of PrP^{Sc} that is most favored (i.e., class I) and that prions tend to evolve towards this conformation. The observation that 7D emerges at a greater frequency at high titer compared to passage at low titer is reminiscent of the emergence of HY from a mixture of HY and DY where the relative

onset of replication in a common population of neurons determines the emergence of a strain from a mixture (Weissmann 2012).

Interspecies transmission can result in a failure of the newly formed PrP^{Sc} to adapt to the new host species, and these non-adaptive prions may represent a new fourth class of prion strains. Structure of the β2-α2 loop of PrP has been linked to susceptibility to prion infection, with a rigid β2-α2 loop observed to increase susceptibility or resistance to prion infection (Christen et al. 2009; Gossert et al. 2005; Khan et al. 2010; Kurt et al. 2015; Perez et al.; 2010; Sigurdson et al. 2011, 2009, 2010). To clarify the role of β2-α2 loop rigidity in prion conversion, transgenic mice expressing horse PrP (TgEq) were generated (Bian et al. 2017). A large species barrier was observed following Inoculation of TgEq mice with a variety of prions (e.g., CWD, TME, scrapie). Only scrapie isolate SSBP/1 transmitted to TgEq mice (Eq-SSBP/1) led to clinical disease, albeit with low attack rate consistent with interspecies transmission. Interestingly, a second passage of Eq-SSBP/1 in the same TgEq mice did not result in shortening of the incubation period or increase in attack rate, as is expected with serial passage in the same host. Instead, clinical disease or presence of PrP^{Sc} in the CNS was not detected in the inoculated TgEq mice. However, when passaged in transgenic mice expressing ovine PrP, Eq-SSBP/1 established infection in all mice inoculated. Thus, Eq-SSBP/1 could replicate in but not adapt to TqEq mice, instead retaining pathogenicity in TgOv mice, expressing PrP from the original host species from which SSBP/1 was isolated. This non-adaptive prion amplification (NAPA) was also observed following transmission of transmissible mink encephalopathy prions to mice expressing cervid PrP.

The mechanisms responsible for class II prions may explain the inability of NAPA class IV prions to propagate in the new host species. Deformed templating and conformational selection are hypothesized to mechanistically explain class II strains and is predicated on the assumption that all of the newly generated PrP^{Sc} in the new PrP background is infectious for the new PrP sequence. The identification of NAPA class IV strains expands the conformational selection model to include generation of conformations of PrP^{Sc} that are a mismatch for the same PrP sequence (Fig. 4). This observation may further refine the understanding of interspecies transmission. For example, interspecies transmission can result in animals that develop disease after an extended incubation period with levels of PrP^{Sc} in the CNS comparable to fully adapted strains, yet subsequent passage in the same host species results in an extended incubation period compared to the strain that eventually emerges. Previously, this has been interpreted to be a result of strain interference that assumed all of the PrP^{Sc} observed upon interspecies transmission was compatible with the new host PrP. NAPA class IV strains provide a more nuanced interpretation. It is possible that a subpopulation of PrP^{Sc} produced upon interspecies transmission are NAPA class IV prions and that the reason for the extended incubation period upon second intraspecies transmission could be due to strain interference and the presence of NAPA class IV strains that do not contribute to disease. It is unknown if NAPA class IV prions can interfere with the replication of non-NAPA class IV prion strains. Expanding upon this concept, it is theoretically possible that replicative, non-toxic forms of PrP^{Sc} are generated and participate in the species barrier effect (Wang et al. 2017). Overall, the identification of NAPA class

IV prion strains has further refined the understanding of class II strains and the zoonotic potential of prions.

Conclusions

The historical work in the prion field defined the properties and behavior of prion strains. Evidence of phenotypic differences in prion disease was first observed in the natural host; however, transmission to rodents provided unequivocal evidence for prion strain diversity. Rodent studies greatly accelerated the pace of research and led to the identification of the agent and host parameters that influence strain properties including the discovery of *Sinc*. It was found that *Sinc* has a major impact on disease pathogenesis that led to the identification of the three groups of prion strains. These seminal studies placed boundaries on the nature of the informational molecule that encoded prion strain diversity. The informational molecule was heritable as, for example, class I strain properties are stable over a wide variety of transmission conditions. The informational molecule can gradually evolve (adapt) over several passages to a new host or can rapidly mutate in the same host, as evidenced by class II and III strains, respectively. These observations were consistent with a conventional pathogen; however, other findings from the classical period were more difficult to reconcile with a viral etiology. *Sinc* had a profound effect on the outcome of disease, and the observation of overdominance suggested that the host protein encoded by *Sinc* was a component of the agent. In every example, class III strains always resulted in the breakdown to 7D, and, importantly, 7D did not revert back to 87A or another prion strain. The hypothesis proposed for this observation was that the agent lost the nucleic acid information encoding 87A. If mutations in nucleic acid genomes are random, it was unclear why other strains were not identified, and if 87A has lost nucleic acid information, then what was encoding the strain properties of 7D? These observations were difficult to explain with a viral etiology; however, it was also unclear how a protein only agent, prion, could explain these findings.

Prion strain diversity is encoded by the conformation of PrP^{Sc} and provides a mechanistic framework to understand prion strain biology. As discussed in the previous section, the classes of prion strains, the repeated reisolation of the same strains, and the breakdown of 87A to only 7D mechanistically can be explained by deformed templating, PrP^{Sc} conformational selection, and the favored PrP^{Sc} conformation hypothesis. Overdominance is the result of selective incorporation or allelic interference of PrP^{C}, and prion strain interference is competition for the limiting resource, PrP^{C}. Synthetic prions are providing a powerful trackable system to investigate the parameters and mechanisms of prion conversion and strain diversity. High-resolution structural images of PrP^{Sc} are just beginning to provide insight into prion conversion and stain diversity with the ultimate goal of determining the relationship between the strain-specific structures PrP^{Sc} and the phenotype of disease. Once seemingly impossible, the prion hypothesis is compatible with how a protein-only infectious agent can perform complex biological functions, including strain diversity.

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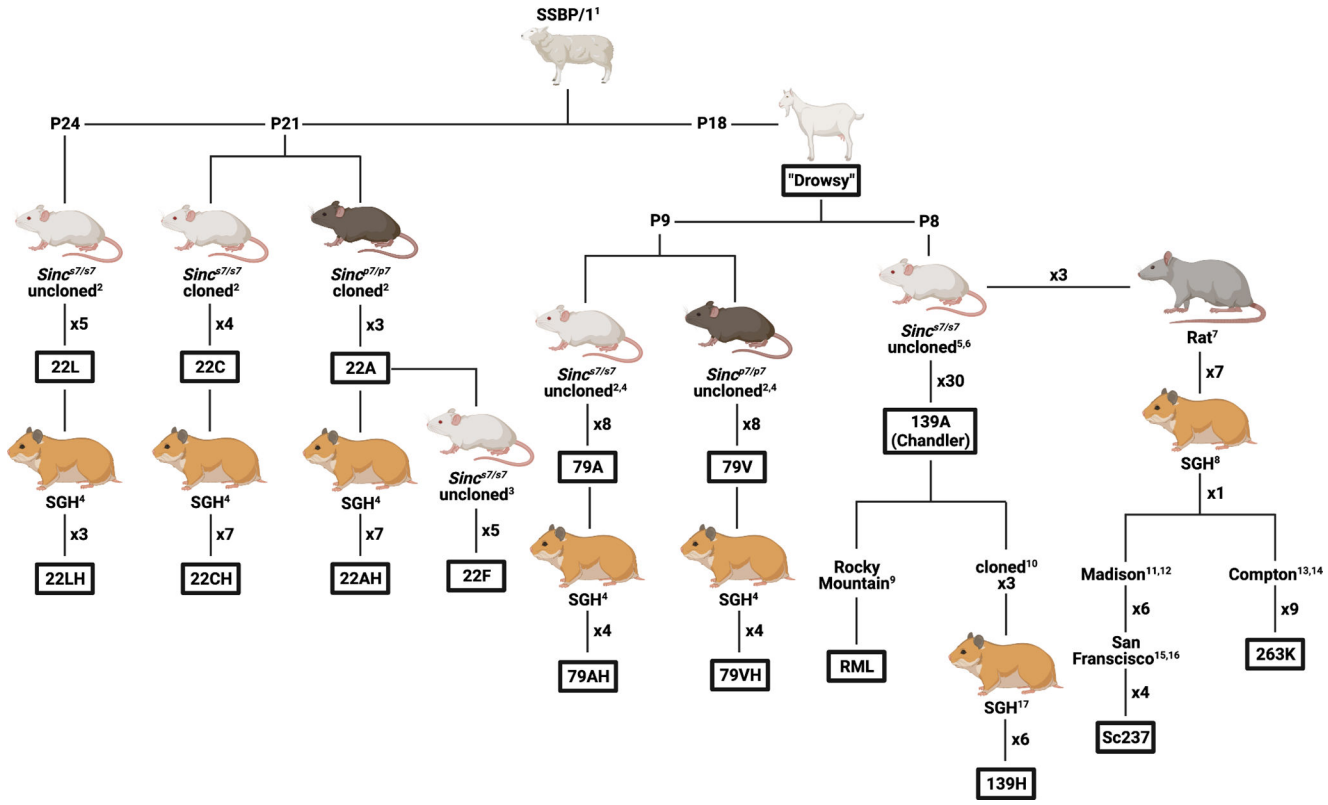

Fig. 1.

Passage history of SSBP/1 in rodents. Transmission of SSBP/1 scrapie to rodents led to isolation of several distinct rodent-adapted strains. There are two main branches: the sheep branch, where sheep scrapie was directly passaged to mice, and the “drowsy” goat branch, where sheep scrapie was passaged through goats prior to passage in mice. P# (e.g., P18) indicates the passage number at which an interspecies transmission event occurred. x# (e.g., × 5) indicates the number of serial passages within a single host species. Strains isolated indicated by an outlined text box. Figure created with BioRender. com. [1]Wilson et al. (1950); [2]Dickinson (1976); [3]Bruce and Dickinson (1979); [4]Kimberlin et al. (1989); [5]Chandler (1961); [6]Chandler and Fisher (1963); [7]Chandler and Fischer (1963); [8]Chandler and Tufrey (1972); [9] Hadlow; [10] Clarke; [11]Kimberlin and Marsh (1975); [12]Marsh and Kimberlin (1975); [13]Kimberlin and Walker 1977; [14]Kimberlin and Walker (1978); [15]Prusiner et al. (1980); [16]Scott et al. (1989); [17]Kimberlin et al. (1987)

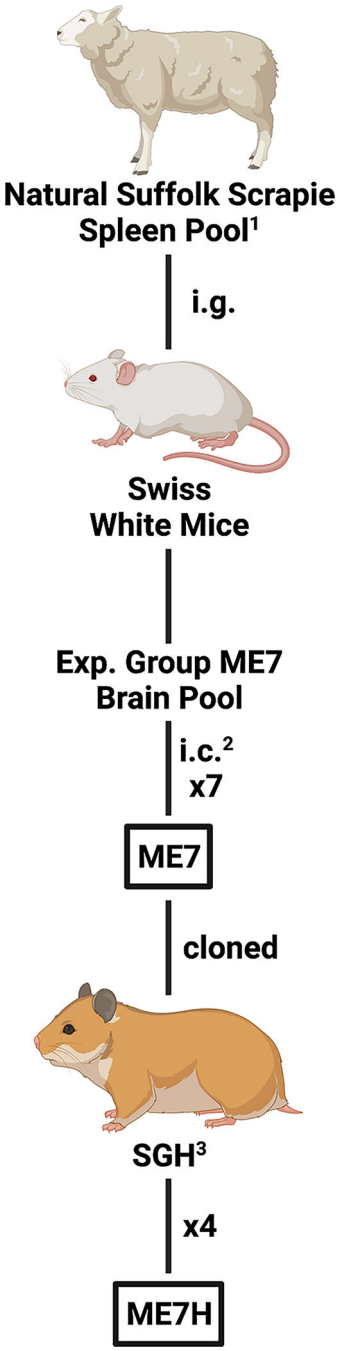

Fig. 2.

Isolation of murine strain ME7 from natural scrapie. Zlotnik ▸ and Rennie were the first to successfully transmit natural Suffolk sheep scrapie to mice (Zlotnik and Rennie 1963). The strain that emerged, designated ME7, is the strain most often isolated following transmission of scrapie to mice. i.g., intragastric inoculation route; i.c., intracranial inoculation route; Exp., experimental. x# (e.g., × 5) indicates the number of serial passages within a single host species. Strains isolated indicated by an outlined text box. Figure created with

BioRender.com. [1]Zlotnik and Rennie (1963); [2]Zlotnik and Rennie (1965); [3]Kimberlin et al. (1989)

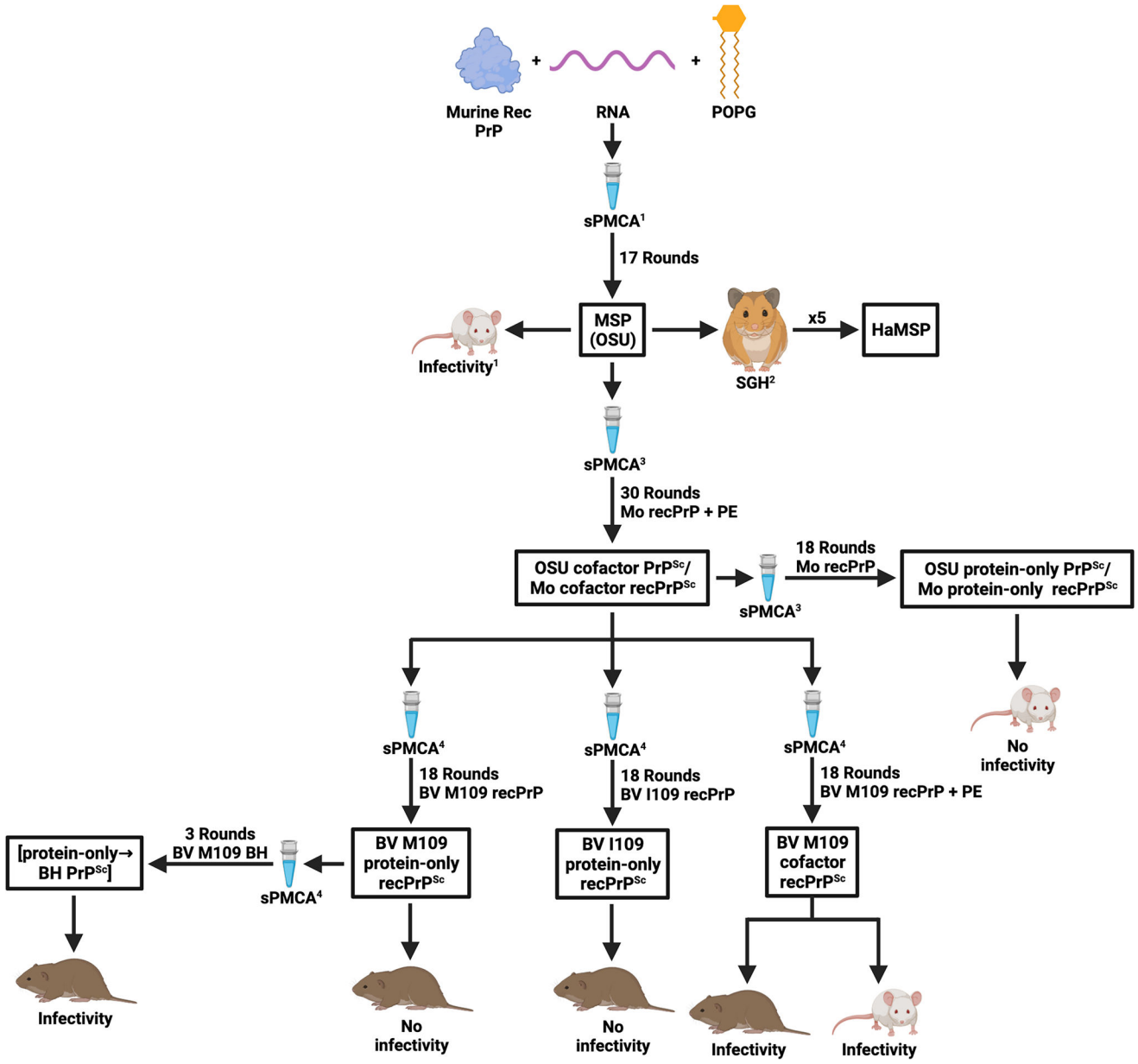

Fig. 3.

Murine synthetic prions (MSP) experimental history. The synthetic prions generated in PMCA with murine recombinant PrP, RNA, and lipid POPG (1-palmitoyl-2-oleoylphosphatidylglycerol) have been used in numerous in vitro and in vivo experiments. MSPs were found to be infectious in both mice and hamsters, the latter demonstrating synthetic prions can cross the species barrier in vivo. Serial PMCA (sPMCA) with a single cofactor, PE (phosphatidylethanolamine), resulted in Mo or BV M109 cofactor recPrP^{Sc} which has in vivo infectivity. Cofactor withdrawal experiments resulted in the formation of Mo or BV M/I109 protein-only recPrP^{Sc}, which lacked in vivo infectivity. sPMCA of BV M109 protein-only recPrP^{Sc} in the presence of BV M109 brain homogenate (BH) restored in vivo infectivity ([protein-only→BH PrP^{Sc}]). OSU, Ohio State University strain; HaMSP, hamster-adapted murine synthetic prions; Mo recPrP, murine recombinant PrP; BV recPrP,

bank vole recombinant PrP. Figure created with BioRender.com. [1]Wang et al. (2010); [2]Block et al. (2021); [3]Deleault et al. (2012a); [4]Burke et al. (2019)

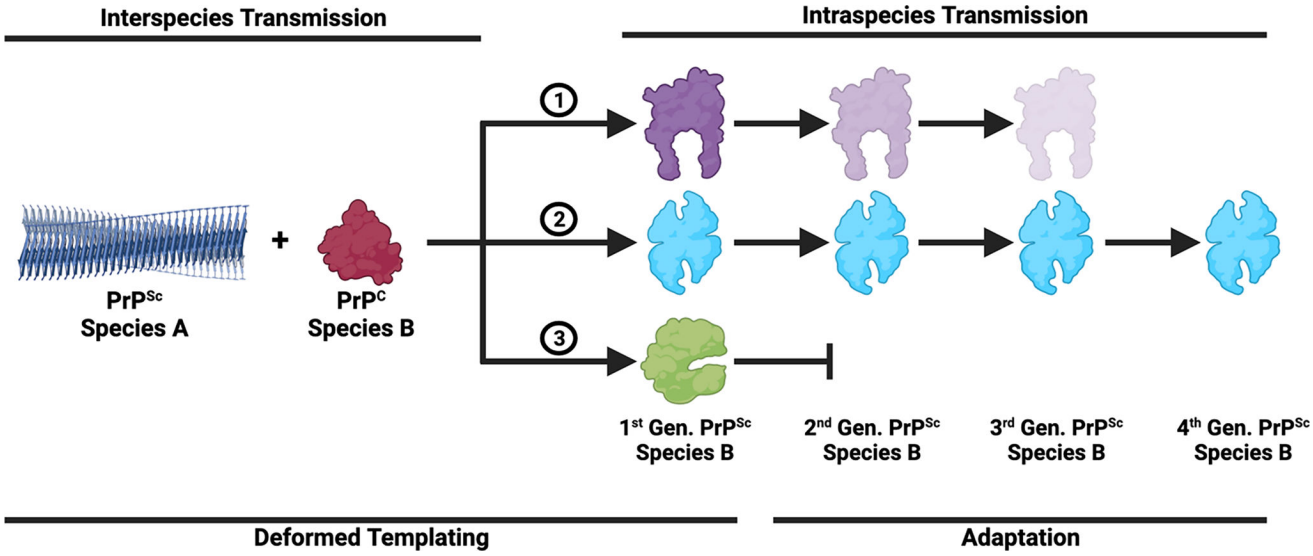

Fig. 4.

Interspecies transmission can lead to generation of PrP^{Sc} conformational mutants. Mismatch in the amino acid sequence between PrP^{Sc} from one species and PrP^{C} from another species during interspecies transmission can result in generation of mutants via deformed templating. The fate of conformational mutants upon serial passage varies. During adaptation, mutants (1) may be compatible for the new host but are outcompeted by another mutant for $PrP.^{C}$, (2) may outcompete other mutants and emerge as the dominant strain, or (3) may be incompatible with the new host. Incompatible mutants may be able to replicate upon initial passage in the new host but never adapt on further passage (non-adaptive prion amplification, NAPA). Gen., generation. Figure created with BioRender.com

Table 1

Biological stability strain classes

Strain class	Definition	Representative strains	References
I	Stable; retain strain identity irrespective of host PrP genotype/species	ME7, 22C, 139A	Bruce and Dickinson (1979); Dickinson (1976); Dickinson et al. (1986)
II	Stable within PrP genotype in which isolated; transmission to different genotype results in adaptation and emergence of new strain	22A	Bruce and Dickinson (1979); Fraser (1979); Dickinson and Outram (1979); Dickinson et al. (1986)
III	Unstable; loses strain identity during passage in isolation PrP genotype, abrupt change to class I strain	87A (31A, 51C, 125A, 138A)[a]	Bruce and Dickinson (1979); Dickinson et al. (1986)
IV	Stable within PrP genotype in which isolated; transmission to different genotype results in no adaptation, retains pathogenicity for original host	EqSSBP/1 (SSBP/1 in TgEq mice[b])	Bian et al. (2017)

[a]These strains are indistinguishable from 87A

[b]Mice that express horse PrP

Table 2

Methods to generate synthetic prions

Generation conditions	Species/PrP	Bioassay	Strain	Comments	References
Denaturing					
Monomeric recPrP folded into fibrils (incubation with urea in buffer), fibrils used as seed	Mouse; recPrP(89–230) expressed in *E. coli*	Tg9949[a], Tg4053[b], FVB mice	MoSP1	First synthetic prions	Legname et al. (2004); Legname et al. (2005)
Folded into amyloid (incubation with urea in buffer)	Mouse; recPrP(23–230) expressed in *E. coli*	Tg9949, Tg4053	MoSP2, 3, 4	Protease-sensitive synthetic prions	Colby et al. (2010)
Folded into amyloid (incubation with urea in buffer); urea, salt, pH, and temperature varied	Mouse; recPrP(23–230) or recPrP(89–230) expressed in *E. coli*	Tg4053, FVB; MoSP8 lacked in vivo infectivity	MoSP5, 6, 7, 8	Demonstrated generation conditions affect strain properties	Colby et al. (2009)
Annealing					
Folded into fibrils (incubation with Gdn-HCl and urea in buffer); fibrils annealed in presence of NBH[c]	Hamster recPrP(23–231) expressed in *E. coli*	Syrian hamsters	SSLOW	Distinct neuropathology (large plaques); basis of the deformed templating model	Makarava et al. (2010)
Folded into fibrils (incubation with Gdn-HCl and urea in buffer); fibrils annealed in the presence of BSA[d]	Hamster recPrP(23–231) expressed in *E. coli*	Syrian hamsters	LOTSS	No clinical disease, but presence of PrPres and neuropathology (similar to SSLOW)	Makarava et al. (2011)
PMCA					
Serial PMCA with PrP^C and poly(A) RNA	Purified hamster PrP^C	Syrian hamsters	Unnamed	First synthetic prions generated with PMCA	Deleault et al. (2007)
Serial PMCA with recPrP, RNA, POPG[e]	Murine recPrP(23–230) expressed in *E. coli*	CD-1 mice; Syrian hamsters	OSU/MSP; HaMSP	First synthetic prions to cross species barrio in vitro and in vivo	Wang et al. (2010); Burke et al. (2019); Block et al. (2021)

[a] PrP expression 16x WT

[b] PrP expression 4-8x WT

[c] *NBH* normal brain homogenate from hamster

[d] *BSA* bovine serum albumin

[e] *POPG* 1-palmitoyl-2-oleoylphosphatidylglycerol