Prions on the move

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Prions consist mainly, if not entirely, of PrP^{Sc}, an aggregated conformer of the host protein PrP^C. Prions come in different strains, all based on the same PrP^C sequence, but differing in their conformations. The efficiency of prion transmission between species is usually low, but increases after serial transmission in the new host, suggesting a process involving mutation and selection. Even within the same species, the transfer of prions between cell types entails a selection of favoured 'substrains', and propagation of prions in the presence of an inhibitory drug can result in the appearance of drug-resistant prion populations. We propose that prion populations are comprised of a variety of conformers, constituting 'quasi-species', from which the one replicating most efficiently in a particular environment is selected.

Keywords: mutation; quasi-species; seeding hypothesis; selection; strain

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See Glossary for abbreviations used in this article.

Introduction

Prions are infectious agents that cause transmissible spongiform encephalopathies (TSEs). They are comprised mainly, if not entirely, of PrP^{Sc}, an aggregated conformer of the normal host glycoprotein, PrP^C (Prusiner, 1991). Propagation of PrP^{Sc} occurs through seeded polymerization, a process by which PrP^C is recruited into the PrP^{Sc} aggregate, and adopts its 'abnormal' conformation (Gajdusek, 1988; Jarrett & Lansbury, 1993). PrP^{Sc} has physicochemical properties that distinguish it from PrP^C, such as aggregation, precipitability by NaPTA and exposure of distinctive epitopes. We distinguish between rPrP^{Sc}, which is largely resistant to proteinase K, and sPrP^{Sc}, which is susceptible to proteinase K (Cronier *et al*, 2008; Nazor *et al*, 2005; Safar *et al*, 1998; Tzaban *et al*, 2002) but resistant to pronase E digestion (D'Castro *et al*, 2010). Both forms of PrP^{Sc}, but not PrP^C, are precipitable by NaPTA (Safar *et al*, 1998).

The mature form of the murine PrP protein consists of 208 amino acids, comprises two potential N-glycosylation sites—which might or might not carry complex glycans—and is attached to the outer surface of the plasma membrane by a GPI anchor. PrP is encoded by a single-copy gene, known as *Prnp* in mice, which encompasses

a coding sequence devoid of introns. Interestingly, inbred mice homozygous for *Prnp* can indefinitely propagate more than 20 strains of prions (Bruce, 2003). These strains were originally characterized by different incubation times and neuropathology after injection into various mouse lines. Subsequently, it was found that individual strains are associated with physicochemical differences of PrP^{Sc} (Baron *et al*, 2011; Bessen & Marsh, 1992; Collinge *et al*, 1996; Kuczius & Groschup, 1999; Peretz *et al*, 2001a; Telling *et al*, 1996), as well as with distinct tropism for cultured cell lines (Mahal *et al*, 2007). It is currently believed that 'strain-ness' is encoded by the conformation of PrP (Bessen & Marsh, 1992; Collinge *et al*, 1996; Telling *et al*, 1996); the role of glycosylation, if any, is still unclear (Sidebar A; Lawson *et al*, 2005; Piro *et al*, 2009; Tuzi *et al*, 2008; Cancellotti *et al*, 2010).

Many, but apparently not all, mammalian species can develop prion disease, spontaneously or as a consequence of infection. In humans, sporadic Creutzfeldt–Jakob disease—which has a worldwide incidence of about 10^{−6}—is believed to be initiated by spontaneous conversion of PrP^C to PrP^{Sc} (Gajdusek, 1991), as are atypical bovine spongiform encephalopathy (BSE) and atypical scrapie in sheep (Baron *et al*, 2007). Given appropriate conditions, prion disease can spread in a population, as exemplified by scrapie in sheep almost worldwide, BSE in the UK and chronic wasting disease in cervids in the USA.

Transmission barriers

In general, there is a considerable barrier to the transmission of prions between animal species, so that even massive intracerebral inoculation causes disease in only a few animals (low 'attack rate') and/or only after long incubation times, if at all ('transmission barrier'; Collinge, 1999). Importantly, when prions are serially transmitted from initial trans-species recipient to other animals of the same species, attack rates increase and incubation times decrease, reflecting adaptation to the new host (Bruce & Fraser, 1991; Kimberlin & Walker, 1977).

To what is the transmission barrier due? Differences in the PrP sequence between donor and recipient make an important contribution, as shown by several instances in which a barrier was overcome or diminished by replacing the *Prnp* gene of the recipient by its counterpart from the donor (Browning *et al*, 2004; Prusiner *et al*, 1990); this might be referred to as a 'sequence barrier'. Sequence barriers are common between animal species, but can occur within a single species, for example between C57BL/6 and VM mice (Bruce, 1993), the PrPs of which—encoded by *Prnp^a* and *Prnp^b* (formerly *sinc⁵⁷* and *Sinc^{P7}*)—differ by just two residues (Westaway *et al*, 1987). The barrier might,

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Sidebar A | In need of answers

- (i) What role does glycosylation have in specifying 'strain-ness'?
- (ii) What is the atomic structure of sPrP^{Sc} and rPrP^{Sc}?
- (iii) Do features other than conformation underlie strain specificity?
- (iv) Which cellular components enable prion replication?
- (v) What determines cell tropism of prion strains?
- (vi) How do strain mutations come about?

to a large extent, be due to conformational restrictions imposed by sequence differences. However, even if the PrP sequences of a donor and recipient are congruent, transmission barriers can still occur, as in the case of variant Creutzfeldt–Jakob disease prions from humans inoculated into mice that express human PrP^C (Hill *et al*, 1997). This indicates that some components in the recipient that are essential for prion replication—such as 'protein X' proposed by Prusiner and colleagues (Kaneko *et al*, 1997; Telling *et al*, 1995)—might not interact appropriately with the donor's PrP, constituting what might be called a 'cell barrier'. Finally, two prion strains originating from the same donor species, and therefore having the same PrP sequence, might show differing infectivities towards a recipient expressing the same PrP. For example, sporadic but not variant Creutzfeldt–Jakob disease prions are highly infectious to mice transgenic for human PrP (Hill *et al*, 1997), thus exemplifying a 'strain barrier'.

Prion strain mutation and selection *in vivo*

It was observed early on that prions derived from a pool of scrapie-infected sheep brain gave rise to different strains in mice, depending on the genotype of the mice and the passage history of the prions (Bruce, 2003). It was not immediately clear whether this was the consequence of heterogeneity in the original source, mutation during transmission, or both. Prion cloning by endpoint dilution cast light on this question. In this procedure, mice are inoculated with prions at dilutions such that only one in ten or more develop the disease; the transmission is repeated 2–3 times. When uncloned 22C prions raised in *Prnp*^a mice were passaged through *Prnp*^b mice, a new prion strain, 22H, was recovered. However, when cloned 22C prions were subjected to the same procedure, the prion strain remained unchanged, indicating that the uncloned 22C sample had been a mixture of at least two strains (Bruce, 2003). Conversely, transfer of cloned 139A prions from mouse to hamster and back to mouse resulted in a new strain, 139H/M, and this was attributed to mutation of the agent—at the time firmly believed to be a nucleic-acid-containing entity (Kimberlin *et al*, 1987). If a mutation is defined as a heritable change of any replicating entity, as we propose, then the phenomenon observed by Kimberlin was indeed a mutation, even if we now attribute it to a change in the conformation of PrP^{Sc} rather than in the sequence of a nucleic acid.

The adaptation of prions from one host species to another—as reflected by attaining minimal incubation times—usually takes two or more serial transmissions (Bruce & Fraser, 1991). According to the seeding model (Gajdusek, 1988; Jarrett & Lansbury, 1993), the first step in PrP^{Sc} replication requires the addition of host PrP^C to the incoming PrP^{Sc} aggregate. Because the amino acid sequences differ, the host PrP^C might be unable to adopt the conformation required for stable addition to the seed, and a conformational change or mutation of the seed might first be required to allow addition. Such mutations might be favoured in very short seeds, the conformation of which might be less stable than that of longer entities. Alternatively, the host

Glossary

Brain [22L]	brain infected with 22L prions
DAPH-12	4,5-bis-4-methoxyanilinophthalimide
EGCG	epigallocatechin-3-gallate
ELISA	enzyme-linked immunosorbent assay
GPI	glycosylphosphatidylinositol
NaPTA	sodium phosphotungstate
PK1 [22L]	PK1 cells infected with 22L prions
PMCA	protein misfolding cyclic amplification
<i>Prnp</i>	murine prion protein gene
PrP	prion protein
PrP ^C	PrP, cellular (normal) form
PrP ^{Sc}	PrP, scrapie (pathogenic) form
RML	Rocky Mountain Laboratory (prion strain)
rPrP ^{Sc}	proteinase-K-resistant PrP ^{Sc}
sPrP ^{Sc}	proteinase-K-sensitive PrP ^{Sc}

PrP^C might assume a conformation that allows it to add to the seed, but is different from that within the seed; the mutation would then be a consequence of unfaithful replication. Even if endowed with the capacity to propagate, the mutated seed might do so only very slowly in the novel environment, and several rounds of mutation and selection might be required to achieve a replication rate that leads to pathogenesis. An informative experiment, reported by Hill and colleagues, showed that inoculation of Sc237 prions from hamsters into mice could, after two years, lead to accumulation of PrP^{Sc} without causing disease. They also showed that the resulting prion population was able to elicit disease in both mice and hamsters, albeit after long incubation times, but with 100% attack rate (Hill *et al*, 2000). This suggests the slow evolution of a heterogeneous population, comprised of prions able to adapt efficiently to both hamsters and mice. The spectrum of conformations that PrP^{Sc} can adopt in a particular host is constrained by the PrP sequence (Angers *et al*, 2010).

More recently, Prusiner and colleagues generated prions by inoculating mice with polymerized recombinant PrP and found that, upon further transmission to mice, a variety of distinct strains emerged, as assessed by incubation time and conformational stability. Serial propagation of such synthetic prions led to selection of more rapidly propagating strains (Colby *et al*, 2009; Legname *et al*, 2004, 2005, 2006).

In conclusion, there is clear evidence from animal experiments that naturally occurring prions are subject to mutation and selection.

Prion strains in cultured cells

It was recognized early on that most cell lines are not susceptible to chronic infection by prions, and those that are, are often susceptible to some strains and not others (Bosque & Prusiner, 2000; Lehmann, 2005; Nishida *et al*, 2000; Rubenstein *et al*, 1992; Schatzl *et al*, 1997; Vorberg *et al*, 2004). The capacity of a strain to infect a cell line can be determined by the standard scrapie cell assay (SSCA): cells are exposed to a serial dilution of prions, propagated for three splits, and the proportion of PrP^{Sc}-accumulating cells is determined by an ELISA (Fig 1). The infectivity of a sample is expressed as the 'response index', namely the reciprocal of the dilution giving rise to a specified proportion of PrP^{Sc}-positive cells (Klohn *et al*, 2003). Making use of the differential cell tropism of prion strains, Mahal and colleagues developed the cell panel assay (CPA), which allows the characterization of several prion strains, in particular 22L, RML, ME7 and 301C, on a panel of four cell lines, PK1, CAD, R33 and

Fig 1 | The SSCA. Typically, a sample is serially diluted 1:10 and added to monolayers of susceptible cells in 96-well plates for 3 days. The cells are subjected to three 1:10 splits, 20,000 cells are filtered onto plates and rPrP^{Sc}-containing cells are identified by subjecting them to proteinase K treatment followed by an enzyme-linked immunosorbent assay. A plot of infected cells ('spots') versus the logarithm of the dilution allows quantification (Klohn *et al*, 2003; Mahal *et al*, 2007). SSCA, standard scrapie cell assay.

LD9; strains are characterized by their differential ability to infect the various cell lines (Table 1; Mahal *et al*, 2007). Another characteristic parameter is the extent to which chronic infection of PK1 cells is inhibited by swainsonine (swa), an inhibitor of α -mannosidase II

Table 1 | Susceptibility of various cell lines to murine prion strains

	CAD	LD9	PK1	PK1 + swa	R33
22L	+	+	+	+	+
RML	+	+	+	-	-
ME7	+	+	-	-	-
301C	+	-	-	-	-

Susceptibility was assessed by the SSCA (standard scrapie cell assay). The data are simplified from Mahal *et al* (2007) and Browning *et al* (2011).

that causes misglycosylation of N-glycosylated proteins. A typical response to swa is two logs of inhibition for RML and 79A, one log for 139A and none for 22L (Browning *et al*, 2011).

Selection of prion substrains

Brain-derived 22L prions can chronically infect PK1 cells in the presence of swa ('swa-resistant') and R33 cells ('R33-competent'); however, when transferred to cultured cells these properties change. To monitor the adaptation of prions to cell culture, PK1 cells were exposed to 22L-infected brain homogenate, and the prions produced were analysed by using the CPA at various times after infection (Fig 2A). After 9 days—the earliest time point examined (P0)—the prion population was still R33-competent and largely swa-resistant, but after 13, 27 and 40 doublings (P4, P8 and P12, respectively), it was fully R33-incompetent and swa-sensitive—that is, 'PK1-adapted'. Because these changes in properties occur gradually, it seems likely that they reflect the adaptation of the 22L prion population from the brain to the PK1 cell environment, rather than being due to recruitment of some cell-specific components, which would have resulted in an immediate change. Indeed, when the PK1-adapted prions were again propagated in the brain, they reacquired their initial properties as seen in the CPA and elicited histopathology indistinguishable from that of 'authentic' brain-derived 22L prions (Li *et al*, 2010). A likely explanation for these observations is that a prion strain population is a quasi-species (Eigen, 1971), consisting of a major component and many variants, which are constantly being generated and selected against in a particular environment, as described earlier for RNA viruses and retroviruses (Domingo *et al*, 1978, 2006) and also proposed for prions ('cloud model' in Collinge & Clarke (2007); Li *et al*, 2010). When changing hosts, a different variant, or 'substrain', might be selected, and the composition of the population could change accordingly (Fig 2A).

To analyse whether there is a similar change in properties observed in the CPA when transferring 22L prions to a different cell line, R33 cells were inoculated. Both PK1 and R33 cells were derived from murine N2a cells; PK1 was selected for high and R33 for low susceptibility to RML prions (Klohn *et al*, 2003). In this case, no significant change in the properties of the 22L was detected, even after 80 doublings (Mahal *et al*, 2010); retention of R33 competence was not surprising, as it was a condition for survival in the new environment, but swa resistance was also retained. However, when the prions were transferred from the R33 cells to PK1 cells, they gradually became swa-sensitive and R33-incompetent, as they had when they were transferred directly from the brain to PK1 cells. By contrast, when transfer and propagation were carried out in the presence of swa, the prions not only remained swa-resistant, as might be expected, but also continued to be R33-competent, again suggesting that the two properties were linked (Mahal *et al*, 2010).

Fig 2 | 22L prions adapt to their environment. (A) Top: PK1 cells were infected with brain-derived 22L prions containing a majority of 'brain-competent' prions (blue) and multiple 'variant' prions (red, green, yellow and grey)—of which one species is 'PK1-competent, swa-sensitive' (orange)—and passaged for 12 1:10 splits. With successive passages (P0 to P12), the PK1-competent prions are selected and become the main component of the quasi-species population. Bottom: The serial dilutions of the prion samples from the splits indicated were assayed on PK1 cells, PK1 cells + swa and R33 cells by the SSCA (standard scrapie cell assay). (B) Chronically 22L-infected PK1 cells were propagated for ten 1:20 splits in the absence of swa (bottom row, C0 to C10); the 'PK1-competent, swa-sensitive' prions (orange) remained the dominant population. When propagated in the presence of swa (top row, S1 to S10), the prion population (orange) decreased markedly in S1, followed by a gradual increase of swa-resistant (red) prions. When, after five splits in the presence of swa, the population was further propagated in the absence of swa (middle row, SC6 to SC10), the 'PK1-competent, swa-sensitive' prions (orange) eventually became dominant, presumably because they have a slight growth advantage over the other prions (red) in the absence of swa. swa, swainsonine.

We assume that prion properties such as cell tropism and response to swa are encoded in the conformation of PrP^{Sc}. Whether this conformation is modulated by other cellular components—such as small RNAs—remains unknown. Notably, some groups describe a

requirement of RNA or poly(rA) for the PMCA-mediated generation of infectious prions from purified PrP^C or recombinant PrP (Deleault *et al*, 2003, 2007; Wang *et al*, 2010), whereas others do not see this requirement (Kim *et al*, 2010). How can PrP^{Sc} conformation encode

substrain characteristics such as cell tropism? The level of prions in a cell is determined by the rate of synthesis on the one hand and the rate of depletion by partitioning, secretion and degradation on the other. Only if the rate of synthesis is equal to or exceeds that of depletion can chronic infection occur. The structure of PrP^{Sc} is probably an important determinant for the relevant rate constants, in particular of synthesis and degradation. As mentioned above, different strains of PrP^{Sc} show distinct physicochemical properties. However, we have not detected differences in the conformational stability or susceptibility to degradation of PrP^{Sc} associated with substrains, probably because these differences are too discrete to be detectable by relatively crude methods.

Selection of drug-resistant mutants

Can the swa-sensitive PK1-adapted 22L prion population convert to swa resistance when propagated in the presence of swa? After one passage (S1) in the presence of swa, the infectivity level of the population dropped precipitously, by more than 90%, but after the second passage, infectivity increased and the prions were fully swa-resistant when assayed on PK1 cells (Fig 2B). After the eighth passage (S8), the level of infectivity was as high as before the propagation in swa, and the prions were swa-resistant. To determine whether the transition to swa resistance was reversible, cells that had been passaged five times in the presence of swa (S5) were propagated in the absence of swa: the prions continued to be swa-resistant for four splits (SC9), but after the fifth split they had become swa-sensitive. The conversion to swa resistance required only a few passages because swa-sensitive prions were unable to replicate in the presence of swa, while the reverse process took longer, presumably because the swa-sensitive prions have only a slight replicative advantage over their swa-resistant counterparts.

Is the misglycosylation of PrP^{Sc} caused by swa the process that renders prions resistant to the drug? The answer is clearly no, as 22L prions propagated in the presence of swa (S5) were fully drug-resistant after five splits; when swa was withdrawn, PrP^{Sc} was again normally glycosylated after propagation for two further splits (SC8), but continued to be fully swa-resistant (Li *et al*, 2010).

The 22L prions propagated for ten splits in the presence or absence of swa (S10 and C10, respectively) showed distinctive behaviour not only in their drug resistance, but also in that S10 prions were R33-competent whereas C10 prions were R33-incompetent (Li *et al*, 2010), and are thus strain variants, or substrains. Cell lysate or concentrated conditioned medium from the two variants, containing equal amounts of PrP^{Sc}, were injected into mouse brain and caused disease. Interestingly, the C10 prions, assayed as lysates, had a significantly longer incubation time than their S10 counterparts, which had almost the same incubation time as the 22L brain homogenate. Nonetheless, after passaging through mouse brain, the CPA properties of S10- and C10-derived prions were indistinguishable from those of authentic 22L-infected brain homogenate. In summary, 22L prions propagated in PK1 cells adapt to the cellular environment and in doing so become swa-sensitive and R33-incompetent, unless constrained by the presence of swa, under which conditions they remain more similar to brain prions. When returned to brain, the incubation time for the swa-sensitive prions is longer than that for the swa-resistant prions, perhaps reflecting the time required to select a brain-adapted population. The various transmissions to which 22L prions were subjected, all of which entailed adaptation to a changed environment with the possible exception of brain 22L transferred to R33 cells, are shown in Fig 3.

Fig 3 | 22L prions transferred into various environments acquire distinct properties. (A) 22L prions from brain (blue) gradually become swa-sensitive and R33-incompetent (orange) when passaged in PK1 cells, and (B) regain their original properties when returned to brain (Li *et al*, 2010). (C) 22L prions from brain (blue) were transferred to R33 cells (green) and remain swa-resistant and R33-competent; it is not clear whether other properties change (Mahal *et al*, 2010). (D) R33-adapted prions (green) when transferred to PK1 cells in the absence of swa, become swa-sensitive and R33-incompetent (orange), but when (E) transferred to PK1 cells in the presence of swa, they remain swa-resistant and R33-competent (red; Mahal *et al*, 2010). (F) swa-resistant, R33-competent prions from PK1 cells propagated in the presence of swa (red) become swa-sensitive and R33-incompetent (orange) when propagated in PK1 cells in the absence of swa. (H) swa-resistant, R33-competent prions from PK1 cells propagated in the presence of swa (red) have a higher specific infectivity when inoculated into brain than swa-sensitive and R33-incompetent (orange) prions from PK1 cells propagated in the absence of swa. All populations are depicted as quasi-species, comprising one major substrain and a few variants. swa, swainsonine.

Similarly, Ghaemmaghami and colleagues reported that treatment with quinacrine of RML-infected mice resulted at first in a reduction of PrP^{Sc} in the brain, followed by an increase of PrP^{Sc} with moderately diminished conformational stability and distinct conformation-dependent immunoassay properties (Ghaemmaghami *et al*, 2009). Moreover, in RML-infected, non-dividing N2a cells exposed to quinacrine, quinacrine-resistant PrP^{Sc} accumulated; this was not the case in dividing cells, showing that the propagation rate of PrP^{Sc} was lower than the rate of cell division.

Interestingly, the prionogenic domain of the yeast prion Sup35—a protein that is completely different from PrP—also gave rise to novel,

Fig 4 | Determining the proportion of pre-existing drug-resistant prions using a frequency assay. PK1 cells are exposed to prions secreted by PK1 cells chronically infected with 22L in the presence or absence of the drug of interest, and pools of 1,000 infected cells (with drug) or eight infected cells (without drug, +992 uninfected cells) are plated in 96-well plates. Cells are grown to confluence and split 1:10 five times in the continued presence or absence of the drug. In these conditions, any well originally containing one or more infected cells becomes positive in the standard scrapie cell assay (SSCA). In this example, 22 of 87,000 cells became PrP^{Sc}-positive in the presence of the drug, and 32 of 696 cells in its absence. Correcting for the Poisson distribution, about 0.5% of the prions were resistant before exposure to the drug. Modified from (Li *et al*, 2010).

drug-resistant strains when propagated in the presence of the inhibitor EGCG. A second inhibitor, DAPH-12—which acts by a different mechanism—synergized with EGCG to enhance inhibition (Roberts *et al*, 2009; Wang *et al*, 2008).

Heterogeneity of prion strain populations

Does a PK1-cell-adapted 22L prion population, which as a whole is swa-sensitive, contain a low level of swa-resistant variants even before exposure to the inhibitor? To answer this question, we performed a frequency assay (Li *et al*, 2010): PK1 cells were exposed to prions secreted by 22L-infected PK1 cells in the absence or presence of swa and an appropriate number of cells was distributed into individual wells of a 96-well plate. After five splits, any well that had received one or more prion-infected cells became massively infected and could be scored by using the SSCA. From the proportion of infected wells in the swa-exposed and control plates, the abundance of pre-existing swa-resistant prions was calculated to be about 0.5% (Fig 4).

To determine how heterogeneity is generated, eight clones of 22L prions were created by infection at endpoint dilution of swa-sensitive prions secreted by PK1 cells chronically infected with 22L. The clones were serially propagated, and the appearance of swa-resistant mutants was monitored by challenging the prion populations with swa and determining whether the population became swa-resistant; swa-sensitive populations that acquire swa resistance after exposure to swa are designated ‘swa-competent’. In two clones, competence was achieved after 31 doublings, in one after 53 and in two others after 86 doublings, whereas the remaining clones had not become swa-competent at that stage. One of the clones that remained swa-sensitive continued to be swa-incompetent for 116 doublings (Li *et al*, 2010, 2011). These findings imply that some clones have a higher mutation rate than others, and that mutability itself is a variable property of prion sub-strains (Fig 5A). Interestingly, all swa-incompetent clones became indistinguishable from wild-type 22L—that is, swa-resistant and R33-competent—when propagated in mouse brain. This is probably because even prions with a low mutation rate, when replicating for approximately 200 days under strong selection pressure, gave rise to a wild-type 22L population (Li *et al*, 2011).

Closing thoughts

Classical work clearly established that the TSE agent, as it was then called, can undergo mutation and selection when transferred between animal hosts. In fact, these were the properties that served as the main argument in favour of the agent being virus-like (Bruce & Dickinson, 1987), an idea that is still supported by some (Miyazawa *et al*, 2011), despite definitive evidence to the contrary (Castilla *et al*, 2008; Kim *et al*, 2010; Wang *et al*, 2010).

The newer results discussed here show that transfer not only between species, but also between cellular environments within the same species, elicits prion adaptation. We have shown that multiply passaged prion populations are heterogeneous, heterogeneity, importantly, arises in cloned prion populations as they are propagated, and the mutation rate can differ for different clones (Fig 5A; Li *et al*, 2011). Different substrains become major components as the population adapts to the environment (Fig 5B; Li *et al*, 2010). This conforms to the predicted behaviour of a quasi-species, which results when populations have a high mutation rate and constantly eliminate less fit mutants, thereby giving rise to a dynamic steady state of variants.

Fig 5 | Cloned prions give rise to quasi-species populations. (A) During propagation, variant prions are generated by mutation. Mutation rates might differ; for example, swa-resistant prions (red) arise stochastically in clone 1 at stage c, in clone 2 at stage d, and in clone 3 not at all during the observation period (scheme based on the experiment of figure 3A in Li *et al.*, (2010)). (B) Conjectural free energy landscape for prion strains and substrains. Substrains are distinguishable collectives of prions that can interconvert reproducibly and readily because they are separated by activation energy barriers that can be overcome in a particular environment under physiological conditions. Different strains are separated by high-energy barriers that are rarely, if ever, overcome in a particular environment. The extent to which the individual wells are populated (red blocks) is governed by the accumulation rate of the prion substrain in that environment. When the environment changes, such as when prions are transferred between tissues, different substrains might be favoured.

Heterogeneity and the underlying mutations are probably due to changes in PrP^{Sc} conformation; because they arise readily and are reversible in the cases discussed above, we assume that the activation energy required for the transition is low, allowing the formation of a substantial number of distinct conformers. The conjectural free energy landscape shown in Fig 5B depicts two series of wells separated by low activation energy barriers labelled A1–A10 and B1–B8, corresponding to substrains of strains A and B. The two sets of substrains are separated by a high-energy barrier, which prevents interconversion of strains. The extent to which a well is populated, provided it is thermodynamically accessible, depends on the replicative potential of the substrain. When a population is transferred to a different environment, the free energy profile might change, allowing the population of wells that were not accessible in the first environment and leading to a different dominant substrain (Mahal *et al.*, 2010).

How many murine prion strains and substrains can be encoded by a particular PrP sequence? Even if PrP^{Sc} conformations are not modulated by covalently linked glycans or by non-PrP components, their number is probably exceedingly large (Sawaya *et al.*, 2007). In the case of a protein monomer, the activation energy barriers between

different conformational states are so low that only the lowest free energy state is substantially populated at room temperature or above. However, in a multimer consisting of a large number of subunits, a vast number of conformations that would be unstable in a monomer are stabilized because the activation energy required to simultaneously change the conformation of an assembly of monomers is additive or close to additive and exceeds thermal energy at physiological conditions. Thus, prion and virus populations exhibit an unexpected commonality: both are heterogeneous and achieve heterogeneity by mutations, and as a consequence are subject to selection in distinct environments, but virus mutations are due to nucleotide changes and prion mutations to conformational states of a protein.

The conclusion that prions constitute quasi-species populations subject to mutation and selection has an impact on our thinking about therapeutic approaches. Because of a prion's potential to acquire drug resistance, a more effective approach would be to starve it of its precursor, PrP^C, either by abrogating its synthesis at the transcriptional or translational level, or by using a drug or antibody (Enari *et al.*, 2001; Peretz *et al.*, 2001b) to prevent its transport to the site of PrP^C-to-PrP^{Sc} conversion, hinder its conversion and/

or promote its accelerated turnover. PrP depletion has no deleterious effects, at least in mice (Büeler *et al*, 1992; Mallucci *et al*, 2002) and cattle (Richt *et al*, 2007). Drugs directly targeting PrP^{Sc} are more likely to engender resistance, because small conformational differences could abrogate binding; this problem might be countered by the use of two or more drugs with different target sites (Duennwald & Shorter, 2010; Roberts *et al*, 2009). A further therapeutic option would be the induction of autophagy, which has been shown to clear PrP^{Sc} from infected cell cultures (Aguib *et al*, 2009; Heiseke *et al*, 2010). However, it should be noted that prion-infected cell cultures are poor models for therapeutic drugs, because cell division potentially contributes to PrP^{Sc} depletion and thus a modest effect of a drug could lead to clearance of infection, which is not the case with non-dividing neurons in the brain. So far, despite considerable efforts by numerous groups, there is no effective therapy on the horizon.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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