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# Mammalian Prion Biology: One Century of Evolving Concepts

Review

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**Prions have been responsible for an entire century of tragic episodes. Fifty years ago, kuru decimated the population of Papua New Guinea. Then, iatrogenic transmission of prions caused more than 250 cases of Creutzfeldt-Jakob disease. More recently, transmission of bovine spongiform encephalopathy to humans caused a widespread health scare. On the other hand, the biology of prions represents a fascinating and poorly understood phenomenon, which may account for more than just diseases and may represent a fundamental mechanism of crosstalk between proteins. The two decades since Stanley Prusiner's formulation of the protein-only hypothesis have witnessed spectacular advances, and yet some of the most basic questions in prion science have remained unanswered.**

## Introduction

A few years ago, it was memorably stated that prion diseases (also termed transmissible spongiform encephalopathies, or TSE) constitute one of the best-understood groups of neurodegenerative diseases (DeArmond and Prusiner, 1995). Depending on whom you ask, this statement may be regarded as entirely correct or completely off the mark.

Of course, prion diseases are quite well understood. Largely thanks to the enthusiasm and intuition of pioneers such as Stanley Prusiner and Charles Weissmann, progress in prion science has experienced two decades of quantum leaps. These include the isolation of the disease-associated, protease-resistant prion protein, $PrP^{Sc}$ (Bolton et al., 1982), the formulation of the protein-only hypothesis (Prusiner, 1982), the cloning of the *Prnp* gene that encodes $PrP^{C}$ and the startling realization that it is a normal, cellular gene (Basler et al., 1986; Chesebro et al., 1985; Oesch et al., 1985), the discovery that the host-determined aspects of the "species barrier" are crucially governed by the sequence of $PrP^{C}$ (Scott et al., 1989), the linkage between *PRNP* mutations and hereditary prion disease (Hsiao et al., 1989), and the demonstration that $PrP^{C}$-deficient mice are alive and well, but resistant to prion diseases (Büeler et al., 1992, 1993).

And yet one may argue that prions are not well understood at all! We are still unable to precisely pinpoint the physical nature of the agent (Chesebro, 1998), and we do not avail of any high-resolution molecular structure of $PrP^{Sc}$. Hence, the models of conversion of $PrP^{C}$ to $PrP^{Sc}$ are speculative at best, and the conversion process could not be reproduced under cell-free conditions in a way that would lead to replication of prion infectivity. Finally, precious little knowledge is available on how the infectious agent damages the brain, and the function of the normal protein continues to be obscure.

### *The Timeline of TSE Research*

In one or the other form, prions have captured a sizeable mind share for almost two centuries (Table 1). Scrapie—the prototypic prion disease affecting sheep and goat—had been a concern since the 19th century. This is understandable given the importance of the wool textile business in the industrial revolution. But the crucial breakthrough was already achieved in the 1930s by the experimental transmission of scrapie to goats (Cuille and Chelle, 1939). Little happened in the two following decades, until Carleton Gajdusek showed that kuru, which was decimating the aborigines of Papua New Guinea (Gajdusek and Zigas, 1957), was a transmissible spongiform encephalopathy. Interestingly, the first attempts at transmitting kuru to primates failed for the same reason that experimental transmission of scrapie among sheep had failed for decades: the incubation time of the disease was longer than the patience of the investigators (Schwartz, 2003). Following a concise suggestion by William Hadlow that kuru resembled scrapie, and hence might exhibit a very long incubation time (Hadlow, 1959), Gajdusek achieved transmission of kuru to chimps (Gajdusek et al., 1966, 1967) and, shortly thereafter, transmission of Creutzfeldt-Jakob disease (CJD) (Gibbs et al., 1968).

It is remarkable (and somewhat sobering) to note that some of the questions that had already been formulated in the 19th century are still open. For example, is sheep scrapie a predominantly genetic or infectious disease? If the latter is true, how does it spread among flocks? The wildfire-like epizootic of chronic wasting disease in North American cervids (Williams and Young, 1980), as well as the "scrapie eradication plan" of the European Union (which aims at selective breeding of purportedly scrapie-resistant sheep genotypes), bears the most recent witness to the general importance of these issues.

### *The Nature of the Prion*

Throughout this paper, the term "prion" is used to denote the infectious principle active in TSEs. The various hypotheses of TSE pathogenesis state that the prion may be congruent, partially overlapping, or different from the protease-resistant form of PrP found in prion diseases, which is termed $PrP^{Sc}$.

Two papers reprinted in the current issue of *Cell* represent two major turning points in prion research. The first paper describes the discovery, by Stanley Prusiner and coworkers, of a crucial property of the prion: its remarkable resilience against proteolytic degradation (McKinley et al., 1983). Digestion with 50 μg/ml of proteinase K (PK) at 37°C for 2 hr would not degrade the carboxy-proximal domain of $PrP^{Sc}$ nor decrease the infectious titer of the prion preparation. But $PrP^{Sc}$ is not "unbreakable" and can eventually be digested by more vigorous enzymatic treatment—in which case prion infectivity titers will also subside. This remarkable discovery identi-

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Table 1. Essential Chronology of Prion Research

| | |
|---|---|
| Mid 18th Century | Earliest Description of Scrapie Recorded |
| 1898 | Neuronal vacuolation discovered in brains of scrapie-sick sheep |
| 1918 | Contagious spread of scrapie in natural conditions suspected |
| 1920 | First cases of CJD described (Creutzfeldt, 1920; Jakob, 1921) |
| 1937 | Scrapie epidemic in Scotland following administration of formalin-treated louping ill vaccine prepared from sheep brain |
| 1939 | Experimental transmission of scrapie reported (Cuille and Chelle, 1939) |
| 1955–1957 | Kuru discovered among Fore people of Papua New Guinea (Gajdusek and Zigas, 1957) |
| 1959 | Similarities between kuru and scrapie noted (Hadlow, 1959) |
| 1961 | Multiple strains of scrapie agent described (Pattison and Millson, 1961) |
| 1961 | Scrapie transmitted to mice (Chandler, 1961) |
| 1963 | Transmission of kuru to chimpanzees reported (Gajdusek et al., 1966) |
| 1966 | Scrapie agent found to be highly resistant to ionizing radiation and ultraviolet light (Alper et al., 1966, 1967) |
| 1967 | First enunciation of the protein-only hypothesis (Griffith, 1967) |
| 1968 | CJD transmitted to chimpanzees (Gibbs et al., 1968) |
| | Description of *Sinc* gene affecting scrapie incubation period in mice (Dickinson et al., 1968) |
| 1974 | First documented iatrogenic prion transmission (corneal graft) (Duffy et al., 1974) |
| 1980 | Protease resistant, highly hydrophobic protein discovered in hamster brain fractions highly enriched for scrapie infectivity (Prusiner et al., 1980) |
| 1982 | Prion concept enunciated (Prusiner, 1982) |
| 1985 | Gene encoding $PrP^{C}$ cloned (Chesebro et al., 1985; Oesch et al., 1985) |
| 1986 | $PrP^{C}$ and $PrP^{Sc}$ isoforms shown to be encoded by same host gene (Basler et al., 1986) |
| 1987 | Linkage between *Prnp* and scrapie incubation period in mice (Westaway et al., 1987) |
| | First report of BSE in cattle (Wells et al., 1987) |
| 1989 | Mutation in PrP linked to Gerstmann-Sträussler syndrome (Hsiao et al., 1989) |
| | Importance of isologous $PrP^{C}/PrP^{Sc}$ interactions established (Scott et al., 1989) |
| 1992 | Ablation of *Prnp* by gene targeting in mice (Büeler et al., 1992) |
| 1993 | $Prnp^{o/o}$ mice are resistant to scrapie inoculation (Büeler et al., 1993; Sailer et al., 1994) |
| | Structural differences between $PrP^{C}$ and $PrP^{Sc}$ isoforms noted (Pan et al., 1993) |
| 1994 | Cell-free conversion of $PrP^{C}$ to protease-resistant PrP (Kocisko et al., 1994) |
| 1996 | New variant of CJD identified (Will et al., 1996) |
| | BSE prion strain carries a distinct glycotype signature (Collinge et al., 1996) |
| | First NMR structure of core murine $PrP^{C}$ solved (Riek et al., 1996) |
| 1997 | Evidence that nvCJD is caused by the BSE agent (Bruce et al., 1997; Hill et al., 1997a) |
| | B lymphocytes necessary for peripheral prion pathogenesis (Klein et al., 1997) |
| 1998 | Genes controlling incubation period are congruent with *Prnp* (Moore et al., 1998) |
| 1999 | Discovery of the $PrP^{C}$ homolog (Moore et al., 1999) |
| 2000 | Temporary depletion of lymphoid FDCs impairs prion replication (Montrasio et al., 2000) |
| | Experimental transmission of BSE in sheep by blood transfusion (Houston et al., 2000) |
| 2001 | Complement involved in prion pathogenesis (Klein et al., 2001; Mabbott et al., 2001) |
| 2003 | Transgenic expression of soluble PrP inhibits prion replication (Meier et al., 2003) |

fied $PrP^{Sc}$ as the first reliable surrogate marker of prion infection. The impact of this technology was phenomenal: even now, twenty years after its original description, the detection of PK-resistant prion protein (termed $PrP^{27\text{-}30}$ because of its molecular weight after hydrolysis of its PK-sensitive amino-terminal domain) remains the gold standard for biochemical diagnosis of prion diseases and forms the basis for all of the currently marketed BSE tests (Figure 1).

The second paper, to which one of us had the privilege of contributing, verifies a crucial prediction of Prusiner's protein-only hypothesis (Büeler et al., 1993). If $PrP^{Sc}$ multiplies by imparting its conformation onto host-borne $PrP^{C}$, organisms devoid of $PrP^{C}$ should be resistant to prion infection. This idea was compelling, but in the early days of prion research, no technology was available that would allow for the targeted removal of a specific gene from the mammalian genome. As soon as in vivo gene ablation became feasible (Zijlstra et al., 1990), Hansrüedi Büeler and Charles Weissmann set out to ablate the *Prnp* gene, which encodes $PrP^{C}$. $Prnp^{o/o}$ mice were alive and well (Büeler et al., 1992), nowithstanding some minor abnormalities (Collinge et al., 1994; Tobler et al., 1996; Watarai et al., 2003)—some of which may not even be causally related to the prion gene (Aguzzi and Hardt, 2003). The excitement in Zurich was considerable as it became gradually clear that inoculation of $Prnp^{o/o}$ mice with brain homogenate from scrapie-sick mice failed to induce disease of any kind (Büeler et al., 1993) or elicit any subclinical replication of the agent (Sailer et al., 1994).

The study of Büeler and colleagues has sometimes been invoked as the "final proof" of the protein-only hypothesis. That is certainly not the case: the knockout experiment was designed to *disprove* Prusiner's hypothesis—and it would have certainly done so if $Prnp^{o/o}$ mice had developed disease. As always with negative results, alternative interpretations can be offered (Popper, 1991). Those skeptical of the prion hypothesis were quick in pointing out that $PrP^{C}$ may be a receptor for a hitherto unidentified virus, whose ablation would confer antiviral resistance. Yet it is fair to say that the resistance to scrapie of *Prnp* knockout mice constitutes one of the most stringent challenges to the protein-only hypothesis. Hence its failure is very significant.

The availability of $Prnp^{o/o}$ mice has triggered a cascade of technological and conceptual advances. For example, it emerged that $PrP^{C}$, besides controlling prion replication, is necessary for neuronal damage: $Prnp^{o/o}$ neurons adjacent to infected $Prnp^{+/+}$ brain grafts do not

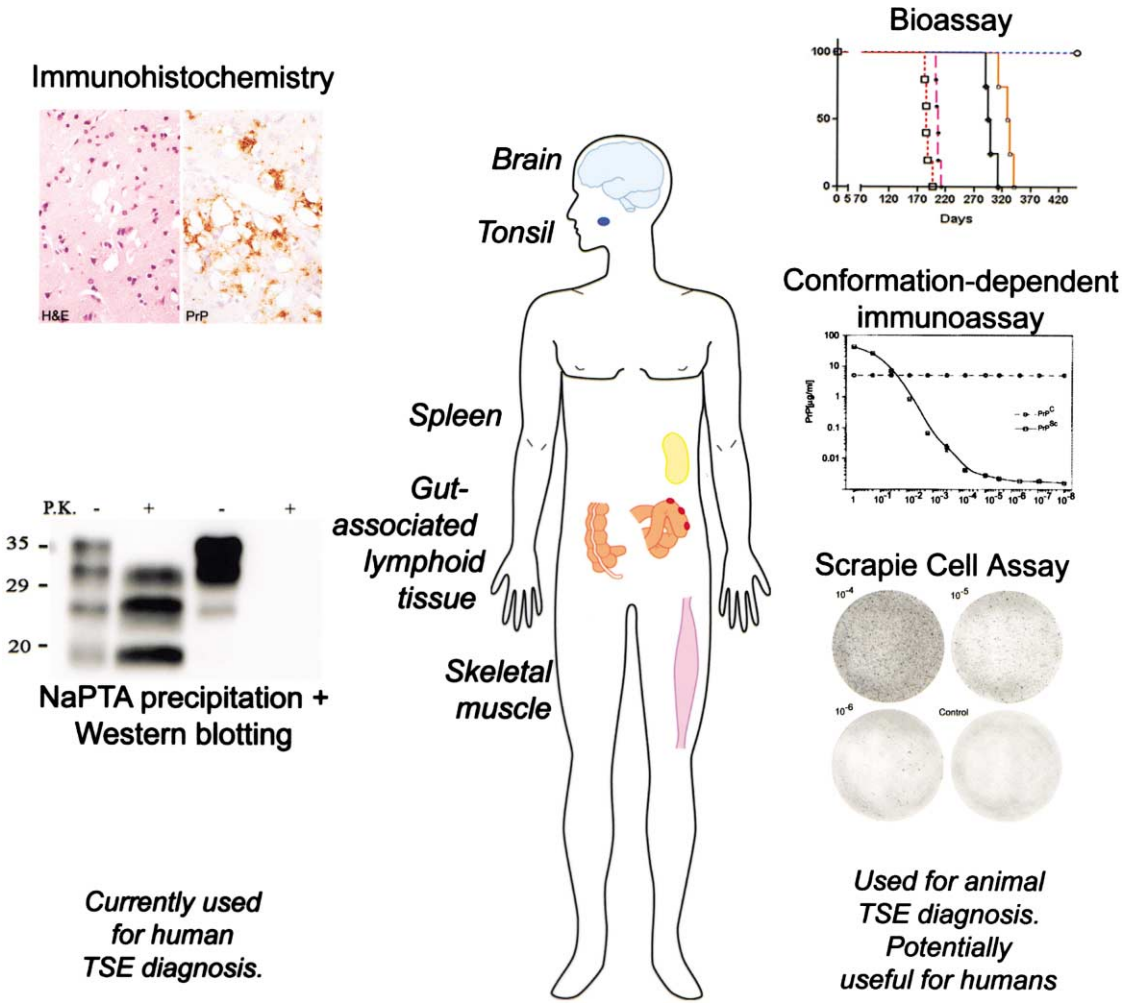



Figure 1. Diagnostic Procedures for Prion Diseases

Synopsis of current diagnostic methods for TSE in humans (left panel) and in experimental animals (right panel). Most methods rely upon the detection of PK-resistant $PrP^{Sc}$. The tissues in which $PrP^{Sc}$ has been detected in humans are listed in the middle panel. While $PrP^{Sc}$ deposits are most abundant in the CNS, the list of peripheral organs in which $PrP^{Sc}$ can be detected has significantly grown in recent years; it now includes most lymphoid organs as well as skeletal muscle.

incur damage (Brandner et al., 1996a). $PrP^{C}$ is also involved in the transport of the infectious agent from peripheral sites to the central nervous system: its expression appears to be needed in a sessile compartment (Blättler et al., 1997), which is likely to be congruent with stromal components of the lymphoreticular tissue (Montrasio et al., 2000) and of the peripheral nervous system (Glatzel et al., 2001). The microenvironment of lymphoid organs appears to control the velocity of neuroinvasion (Prinz et al., 2003a).

$PrP^{C}$ is not only produced by neurons; its expression is in fact quite ubiquitous, notably including lymphocytes (Cashman et al., 1990) and stromal cells of lymphoid organs (Kitamoto et al., 1991). As a result, wild-type mice enjoy an extremely tight immunological tolerance against $PrP^{C}$, which had rendered the production of high-affinity immunoreagents very difficult. Instead, the immunization of *Prnp*$^{o/o}$ mice yielded large numbers of very high-affinity antibodies, some of which form the basis for the current crop of BSE tests.

Still, it proved difficult to generate conformational antibodies discriminating between $PrP^{C}$ and $PrP^{Sc}$. This is surprising in view of the dramatic structural differences between these two isoforms and their differential binding to serum proteins (Fischer et al., 2000). Does the failure of the immune system to generate antibodies specific for $PrP^{Sc}$ indicate that all relevant neoepitopes of $PrP^{Sc}$ that are newly exposed by the conversion of the protein to its disease-associated state are inaccessible? Early claims of discriminatory antibodies, such as Prionics' 15B3 clone (Korth et al., 1997), have not lived up to the expectations. A recently developed antibody against a characteristic tripeptide (YYR) exposed in $PrP^{Sc}$, but not in $PrP^{C}$, may be more promising (Paramithiotis et al., 2003). However, the YYR motif is certainly not specific to $PrP^{Sc}$, and the usefulness of this antibody awaits independent confirmation.

### *The Spontaneous Generation of Prions*

A mesmerizing implication of the protein-only hypothesis is the propagation of prions in an entirely synthetic system. If the infectious agent is a misfolded form of PrP, and its replication is promoted by its interaction

with $PrP^C$, then the entire process should be, in principle, reproducible in a cell-free environment consisting exclusively of $PrP^C$, $PrP^{Sc}$, and maybe some "promoting factors." The importance of such an experiment is immediately evident: de novo generation or amplification of prions from defined components would prove the protein-only hypothesis and set to rest all other alternative explanations (Aguzzi and Weissmann, 1997). Besides, such a system would be extremely valuable for studying the conversion process, for exploring the species barrier phenomenon, and for testing conversion antagonists that may provide therapeutic compounds.

A decisive milestone toward this goal was accomplished with the establishment of an in vitro conversion system based on the coincubation of substantially purified constituents (Kocisko et al., 1994). This seminal work showed that incubation of radiolabeled $PrP^C$ with cold $PrP^{Sc}$ leads to the formation of PK-resistant radiolabeled PrP—indicating that $PrP^{Sc}$ had somehow imparted some of its properties onto $PrP^C$. The original system required vastly superstochiometric amounts of $PrP^{Sc}$, which precluded the detection of any increase in prion infectivity. However, the method was used to probe the conversion efficiency between PrP molecules with different primary sequences and thereby, to some extent, the tightness of species barriers (Bessen et al., 1995; Horiuchi et al., 2000). In the intervening years, the in vitro conversion methodology has yielded remarkable insights and even assays for identification of antiprion compounds.

It was reported that $PrP^{Sc}$ could be amplified by cycles of sonication followed by incubation with brain homogenate (Saborio et al., 2001). The idea behind this experiment was that sonication might fracture large $PrP^{Sc}$ aggregates into smaller units, each one of which would accrue $PrP^C$ and act as independent "infectious unit." Several skeptics, however, have pointed out that this intriguing report is still awaiting independent confirmation. Also, more than two years after its publication, no evidence has come forward that this "protein misfolding cyclic amplification" would augment the infectivity of any given sample.

Along parallel lines, conditions were established at that recombinantly produced PrP was transformed into an isoform termed βPrP, with several typical properties of $PrP^{Sc}$ (Jackson et al., 1999): increased β sheet content, aggregability, and resistance to PK. This molecule was deemed quite interesting for two main reasons. Firstly, one had hoped that immunization of mice with βPrP might give rise to conformation-specific monoclonal antibodies, which would help in discriminating directly between $PrP^C$ and $PrP^{Sc}$. The latter would render obsolete the venerable PK digestion assay and may facilitate the development of higher-throughput $PrP^{Sc}$ immunoassays. Secondly, βPrP might be equivalent to PrP* (Weissmann, 1991), a metastable intermediate postulated to arise during conversion of $PrP^C$ to $PrP^{Sc}$. If so, inoculation of mice with suitable amounts of βPrP might result in the generation of transmissible disease.

At present βPrP has yet to fulfill either of these two expectations. Yet it is not implausible that additional experimentation in specifically devised animal models may change this negative outcome. As with transmission of kuru to chimps, it is advisable to be patient.

### *Form Follows Function*

If the protein-only hypothesis is correct, one could argue that the prion problem is, in essence, one of protein structure. Whether prions multiply by template-directed refolding or by seeded nucleation, certain domains of $PrP^C$ (or the entire protein) would need to rearrange such that the monomeric protein becomes capable of inducing the same change in further $PrP^C$ monomers (Figure 2A). This idea represents the core of the "template-directed refolding" hypothesis, which predicates an instructionist role for $PrP^{Sc}$ onto $PrP^C$. The experimental evidence is compatible with this hypothesis, yet no positive evidence in its favor has come forward.

Alternatively, it has been proposed that $PrP^{Sc}$ exists in a mass-action equilibrium with $PrP^C$. Such equilibrium would be heavily shifted toward the side of $PrP^C$ so that only minute amounts of $PrP^{Sc}$ would coexist with $PrP^C$. If that were the case, $PrP^{Sc}$ could not possibly represent the infectious agent since it would be ubiquitous. According to this "nucleation" hypothesis (Jarrett and Lansbury, 1993), however, the infectious agent would consist of a highly ordered aggregate of $PrP^{Sc}$ molecules. The aggregated state would be an intrinsic property of infectivity: monomeric $PrP^{Sc}$ would be harmless, but it might be prone to incorporation into nascent $PrP^{Sc}$ aggregates (Figure 2B).

Testing these hypotheses requires precise knowledge of the structural features of both $PrP^C$ and $PrP^{Sc}$. To date, such knowledge has not progressed to a state that would allow for resolution of this question. The structure of $PrP^C$ has been studied extensively with high-resolution methods. Both crystallography (Knaus et al., 2001) and nuclear magnetic resonance (NMR) spectroscopy (Riek et al., 1996) have yielded detailed insights into the arrangement of $PrP^C$ at the atomic level. $PrP^{Sc}$, however, has been amenable merely to low-resolution structural methods.

The NMR studies of recombinant $PrP^C$ yielded a big surprise. The amino-proximal half of the molecule is not structured at all, whereas the carboxy-proximal half is globular and contains three α helices (Riek et al., 1996, 1997). This does not mean that the amino terminus must be randomly coiled in vivo: functional studies in transgenic mice imply that the domain comprising amino acids 32-121 carries out important physiological functions (Shmerling et al., 1998). Maybe the flexible tail of $PrP^C$ acquires a defined structure once it reaches its natural habitat on rafts, which are specialized microdomains of the plasma membrane (Naslavsky et al., 1997).

Why wasn't it yet possible to elucidate the structure of $PrP^{Sc}$? As discussed above, prion infectivity can be recovered only from prion-infected mammalian organisms or (in much lesser quantities) from infected cultured cells. In neither case is the purity of the recovered material satisfactory. Moreover, infectivity-associated $PrP^{Sc}$ appears to consist obligatorily of aggregates; disaggregation sterilizes prions (Prusiner et al., 1981). But insoluble aggregates are resilient to most technologies for determination of protein structure; hence all we know is that $PrP^{Sc}$ consists mainly of β-pleated sheet (Caughey et al., 1991) and that $PrP^{Sc}$ aggregates expose a remarkably ordered structure (Wille et al., 2002).

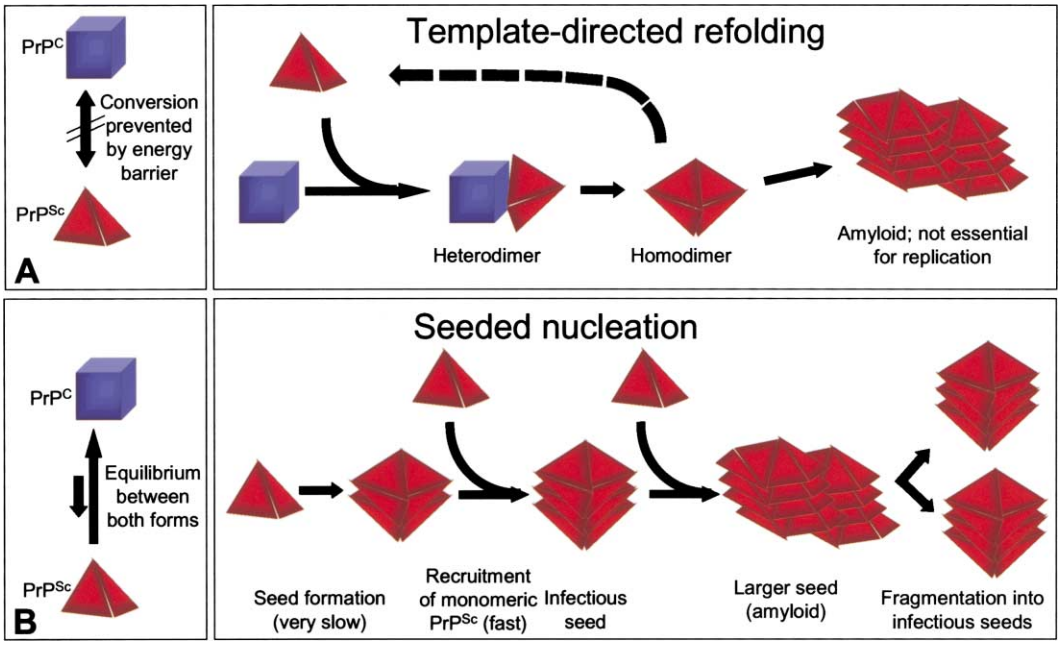



Figure 2. Models for the Conformational Conversion of $PrP^C$ into $PrP^{Sc}$

(A) The "refolding" or template assistance model postulates an interaction between exogenously introduced $PrP^{Sc}$ and endogenous $PrP^C$, which is induced to transform itself into further $PrP^{Sc}$. A high energy barrier may prevent spontaneous conversion of $PrP^C$ into $PrP^{Sc}$.

(B) The "seeding" or nucleation-polymerization model proposes that $PrP^C$ and $PrP^{Sc}$ are in a reversible thermodynamic equilibrium. Only if several monomeric $PrP^{Sc}$ molecules are mounted into a highly ordered seed, further monomeric $PrP^{Sc}$ can be recruited and eventually aggregates to amyloid. Within such a crystal-like seed, $PrP^{Sc}$ becomes stabilized. Fragmentation of $PrP^{Sc}$ aggregates increases the number of nuclei, which can recruit further $PrP^{Sc}$ and thus results in apparent replication of the agent.

### *Yeast Prions*

Thirty years ago, Francois Lacroute described mysterious yeast traits that apparently propagated by nonmendelian genetics (Lacroute, 1971). For two decades, this phenomenon remained unexplained—until Reed Wickner proposed that the unusual genetic properties of these mutants could be explained by a prion-like behavior of two previously identified yeast proteins: Sup35p, an essential component of the translation termination machinery, and Ure2p, a protein that regulates nitrogen metabolism (Wickner, 1994). Further yeast prions were identified in the following, so that one could now argue that the yeast prion phenomenon is much better understood than its mammalian counterpart. The prion-forming domain (PrD) of Sup35p is modular and transferable; artificial prions were generated by fusing a mammalian receptor to the Sup35p PrD (Li and Lindquist, 2000).

In the prion-infected state (termed $\psi^+$), Sup35p is sequestered into fibrils. As consequence, termination of translation is impaired, and reading frames situated downstream of nonsense codons can be translated into proteins (Figure 3). Just like in street traffic, ignoring stop signs does not generally constitute healthy behavior, but Susan Lindquist made a convincing case that such transgressions may play a decisive role in creating "evolutionary buffers." By occasionally switching on bicistronic reading frames through the $\psi^+$ state, yeast cells can reversibly probe the effects of combinatorial expression of mutated genes, hence creating additional layers of evolutionary variation (True and Lindquist, 2000).

### BSE and Other Prion Threats to Humans

When Stanley Prusiner started his first attempts at tackling the problem of TSE (Prusiner et al., 1977), this group of diseases was not exactly in the public limelight. However, bovine spongiform encephalopathy (BSE) was recognized a few years later (Wells et al., 1987) —an event that would dramatically change the public perception of prion diseases. CJD was, and fortunately continues to be, exceedingly rare: its incidence is typically $1/10^6$ inhabitants/year, but reaches $3/10^6$ inhabitants/year in Switzerland, which is currently reporting the highest number of cases (Glatzel et al., 2002, 2003b). Kuru, once decimating the population of Papua New Guinea, has almost disappeared. Iatrogenic transmission of CJD has principally occurred through improperly sterilized neurosurgical instruments, transplants of dura mater, and administration of pituitary hormones of cadaveric origin. While the two latter routes of transmission no longer pose a major threat, a significant number of individuals may have been infected during a critical time window and may develop CJD in the coming years.

Variant CJD (vCJD) has caused some 140 deaths in the United Kingdom and a few cases in France, Italy, and Canada (http://www.doh.gov.uk/cjd/stats/aug02.htm). Epidemiological, biochemical, and histological evidence suggests that vCJD represents transmission of bovine spongiform encephalopathy (BSE) prions to humans (Aguzzi, 1996; Aguzzi and Weissmann, 1996; Bruce et al., 1997; Hill et al., 1997a). The incidence of vCJD in the United Kingdom rose each year from 1996 to 2001, evoking fears of a large upcoming epidemic.

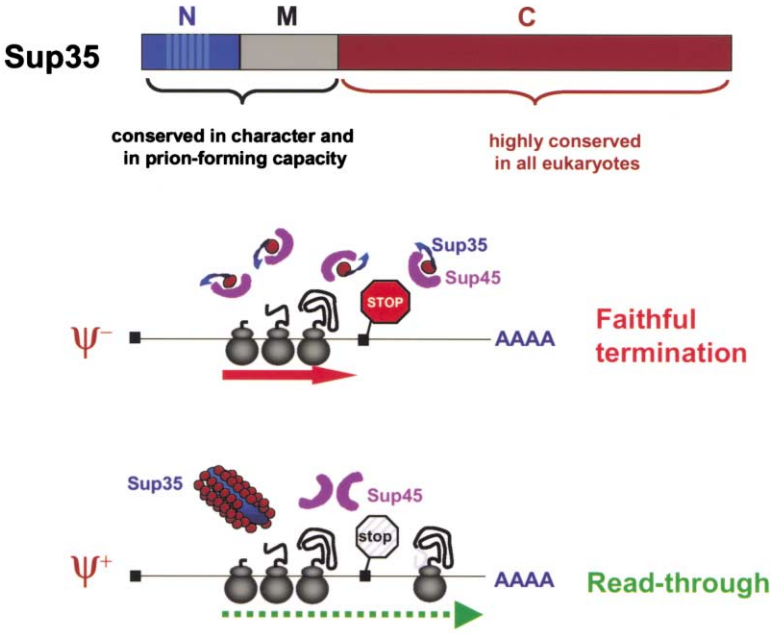



Figure 3. Function of the Yeast Prion, Sup35

(A) Sup35 consists of an amino-terminal glutamine-rich module crucial for conversion into the prion state.

(B) In the $\psi^-$ state, Sup35 is required for reliable termination of translation.

(C) In $\psi^+$ yeast cells, however, Sup35 is sequestered in ordered fibrillary aggregates. Shortage of functional Sup35 leads to transgressions in stop codon recognition and translation of downstream reading frames (red line). In the off state, such pseudogenes may accumulate otherwise toxic mutations. Acquisition of the on ($\psi^+$) state may lead to the appearance of new phenotypes, hence increasing the complexity of genetic variability.

Since the year 2001, however, the incidence of vCJD in the UK appears to be stabilizing (http://www.cjd.ed.ac.uk/vcjdq.htm). One may argue that it is too early to draw any far-reaching conclusions, but each year passing without any dramatic rise in the number of cases increases the hope that the total number of vCJD victims will be limited (Valleron et al., 2001). Presently, there is reason to hope that the incidence of vCJD in the United Kingdom may already be subsiding (Andrews et al., 2003).

vCJD prions accumulate prominently in lymphoreticular tissue, and the latter can be used for diagnostic purposes. Surprisingly, prions accumulate in lymphoid organs and muscle of sporadic CJD patients (Glatzel et al., 2003a).

There is uncertainty surrounding the danger of transmission to humans represented by chronic wasting disease. In fact, even transmissibility of BSE to humans relies on circumstantial evidence. Epidemiology and biochemistry favor the link between BSE and vCJD, but are not ultimately conclusive. The Koch postulates (which would unambiguously assign an infectious agent to a disease) have never been fulfilled, and experimental inoculation of humans was fortunately never performed. Also, accidental exposure to BSE infectivity of a sizable collective at a precisely defined time point has never occurred, or did not result in disease. Likewise, we do not know whether scrapie is just a veterinarian problem that affects only sheep and goat or whether it can cross species barriers and affect humans. Finally, it is unknown whether BSE, upon transmission to sheep, remains as dangerous for humans as cow-derived BSE, or whether it becomes attenuated and acquires the (allegedly) innocuous properties of bona fide sheep scrapie.

## The Elusive Function of $PrP^C$

In spite of the fact that the first *Prnp* knockout mice are available since 12 years (Büeler et al., 1992), the normal function of the cellular prion protein is still unknown. A number of subtle abnormalities have been described in PrP-deficient mice (Collinge et al., 1994; Tobler et al., 1996), but their molecular basis is undefined, and there may be some variability due to the genetic background of the mice utilized. Hence, the only definite phenotype of $Prnp^{o/o}$ mice is their resistance to prion inoculation (Büeler et al., 1993) —yet it seems unlikely that a singular protein that is as highly conserved among species as $PrP^C$, from turtles to frogs, fish, and humans, has evolved for the sole reason of bestowing susceptibility to prion diseases.

If the function of $PrP^C$ were completely unrelated to prion disease pathogenesis, one might argue that $PrP^C$ is just one of many thousands proteins whose function awaits clarification—but why should then the elucidation of the function of $PrP^C$ be given any priority? On the other hand, the function of $PrP^C$ may very well have something to do, in a subtle way, with prion-induced damage. *Prnp* ablation does not elicit disease, even when induced postnatally (Mallucci et al., 2002); hence prion pathology is unlikely to come about by loss of $PrP^C$ function. But assume that $PrP^C$ transduces a signal, or that it possesses some enzymatic activity. If so, conversion to $PrP^{Sc}$ may alter signal transduction strength, or substrate specificity, thereby conferring a toxic dominant function. In these scenarios, understanding the function of $PrP^C$ may help in deciphering prion pathology and maybe even devising therapeutical approaches.

So, what is the evidence that $PrP^C$ may be a signal transducer or an enzyme? Speculations on both hypotheses abound, but facts are scarce. Crosslinking $PrP^C$ with $F(ab)_2$ antibody fragments has been reported to activate intracellular tyrosine kinases (Mouillet-Richard et al., 2000). However, this phenomenon was not reported to occur in vivo, and the only cell line in which it was described was never made available to the scientific community for independent verification. This does not exclude that $PrP^C$ functions as a signal transducer, but the present case is of limited strength.

Is $PrP^C$ an enzyme? Glockshuber noted that $PrP^C$ has similarities to membrane-anchored signal peptidases (Glockshuber et al., 1998), but his observation has not been substantiated by functional data. The speculation that $PrP^C$ may be a superoxide dismutase (Brown et al., 1997, 1999) was perceived as particularly attractive in view of its multiple copper binding sites, and it was recently suggested that amino-proximally truncated $PrP^C$ may depress endogenous dismutase activity (Sakudo et al., 2003). However, $PrP^C$ does not make any measurable contribution to dismutase activity in vivo (Hutter et al., 2003; Waggoner et al., 2000).

Maybe $PrP^C$ and $PrP^{Sc}$ do not possess any intrinsic biological activity, yet they modify the function of other proteins. This supposition has prompted a search for PrP-interacting partners, and there is no dearth of PrP binding proteins: the antiapoptotic protein Bcl-2 (Kurschner et al., 1995), caveolin (Gorodinsky and Harris, 1995; Harmey et al., 1995), the laminin receptor precursor (Rieger et al., 1997), plasminogen (Fischer et al., 2000), and N-CAM (Schmitt-Ulms et al., 2001). None of these interactors, however, have yet revealed a functional pathway in which $PrP^C$ would be involved in vivo. It was recently shown that PrP-deficient macrophages do not support bacterial "swimming internalization" of the Gram-negative bacterium, *Brucella abortus* (Watarai et al., 2003), and that $PrP^C$ interacts with a *Brucella* heat shock protein, Hsp60. These findings raise the question of whether $PrP^C$ may participate in a general Hsp60-dependent "danger sensing" mechanism (Aguzzi and Hardt, 2003).

### *A Doppelganger of the Prion Protein*

The original $Prnp^{o/o}$ mice did not display any severe abnormalities. However, some of the knockout lines generated later, i.e., Ngsk $Prnp^{-/-}$ (Sakaguchi et al., 1996), ZH-II $Prnp^{-/-}$ (Rossi et al., 2001), and Rcm0 mice (Moore et al., 1999), develop progressive cerebellar Purkinje cell degeneration with ataxia in advanced age. This phenotype was originally attributed to the lack of $PrP^C$ and ran counter to the two PrP knockout mouse lines produced earlier: the ZH-I $Prnp^{o/o}$ (Büeler et al., 1992) and the Edbg $Prnp^{-/-}$ mice (Manson et al., 1994). The characterization of Ngsk $Prnp^{-/-}$ mice was particularly conscientious: the authors reintroduced *Prnp* as a transgene by genetic crosses and showed that this manipulation rescued the Purkinje cell degeneration. It seemed entirely reasonable, hence, to conclude that $PrP^C$ is necessary for cerebellar homeostasis. Yet this interpretation could not be easily reconciled with the lack of phenotype in the remaining knockout lines and eventually was proven to be incorrect.

The inconsistency was eventually resolved by David Westaway's discovery of a novel gene located just 16 kilobases downstream of *Prnp* and encoding a 179 residue protein that has sequence similarities to the C terminus of PrP and was thus termed Doppel or Dpl (Moore et al., 1999). It then emerged that the gene targeting strategy in all ataxic PrP-deficient mice was associated with deletion of a splice acceptor site located on the coding exon of *Prnp*. This modification effectively places Dpl under transcriptional control of the *Prnp* promoter. As a consequence, brain expression of Dpl, which is normally very low, skyrockets in Nsgk, ZH-II, and Rcm0 mice (Weissmann and Aguzzi, 1999). This is clearly neurotoxic, as ablation of the Dpl reading frame from ZH-II mice abolishes the Purkinje cell degeneration phenotype (Nicolas Genoud, Axel Behrens, and A.A., unpublished data).

Most intriguingly, Dpl-dependent neurodegeneration is abolished by cell-autonomous coexpression of full-length PrP (Rossi et al., 2001). Formally, this indicates that Dpl and $PrP^C$ act antagonistically, maybe because they bind to a hitherto conjectural common ligand (Figure 4A), which was provisionally termed $L_{PrP}$ (Shmerling et al., 1998). Alternatively, $PrP^C$ and Dpl might engage in heterooligomeric complexes (Figure 4B), whose function could depend on their stoichiometric composition (Behrens and Aguzzi, 2002). The same mechanism may be operative in transgenic mice produced by Doron Shmerling and Charles Weissmann (Shmerling et al., 1998) in an attempt to specify the domain of $PrP^C$ required for prion replication. Expression of a PrP variant that lacks a large part of the N terminus of PrP in $Prnp^{o/o}$ mice induces spontaneous cerebellar degeneration, which however affects granule cells rather than Purkinje cells (the promoter used was inactive in Purkinje cells) and can also be prevented by the coexpression of a single endogenous *Prnp* allele. Structural studies have shown that human Dpl contains a relatively short, flexibly disordered "tail" comprising residues 24-51 and a globular domain extending from residues 52 to 149 for which a detailed structure was obtained (Luhrs et al., 2003). Despite their highly divergent primary sequence, Dpl is largely superimposable to the carboxy-proximal half of $PrP^C$.

The molecular pathways by which Dpl and amino-proximally truncated PrP damage the cerebellum are unknown. However, the suppressibility of both phenotypes by full-length $PrP^C$ is indicative of a high degree of specificity. Therefore, we contend that this model presently represents the best validated window of entry to determine the function of $PrP^C$ in vivo.

## The Basis of Prion Neurotoxicity

$PrP^{Sc}$ accumulation in the brain is the hallmark of prion diseases, and $PrP^{Sc}$ is—for all we know—a major component of the infectious agent. But is $PrP^{Sc}$ also directly responsible for the devastating CNS pathology typical of prion diseases? On the one hand, accumulation of amyloid (or preamyloid) in the CNS is likely to be generally unhealthy, as exemplified by Alzheimer's disease (Aguzzi and Haass, 2003) and cerebral vascular amyloidoses (Revesz et al., 2002). On the other hand, chronic deposition of $PrP^{Sc}$ does not damage *Prnp* knockout brains (Brandner et al., 1996a), and depletion of $PrP^C$ from neurons of scrapie-infected mice prevents disease (Mallucci et al., 2003). Therefore, accumulation of $PrP^{Sc}$ is unlikely to fully account for prion pathology. If so, what is it that actually kills the neurons?

Brains of Creutzfeldt-Jakob disease victims look truly frightening. In heavily affected areas, there is hardly any neuron left, and the brain tissue texture is coarsened by the abnormal growth of astrocytes ("gliosis") and microglial cells. The most telling hallmark is spongiosis, a peculiar microvacuolation affecting residual neural cells.

The molecular steps that emanate from prion replication and lead to such destruction are unknown. Some

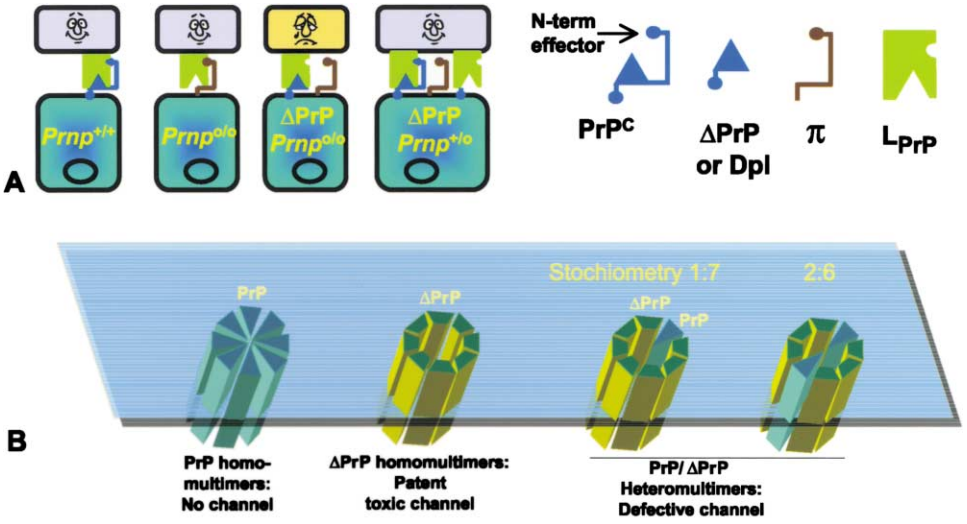



Figure 4. Hypothetical Models for the Function of $PrP^C$ and the Neurotoxicity of $\Delta PrP^C$ and Dpl

(A) $PrP^C$ and Dpl (or ΔPrP) may compete for a common ligand, provisionally termed $L_{PrP}$. In order to accommodate the lack of neurodegeneration in *Prnp*$^{o/o}$ mice, however, one would have to postulate the existence of a functional $PrP^C$ analog, here termed π. While this model accommodates all experimental findings known to date, no physical evidence has come forward for the existence of $L_{PrP}$ and π.

(B) Dpl and ΔPrP may form a homomultimeric toxic aggregate, which may be inactivated by participation of full-length $PrP^C$. Toxicity may come about by various hypothetical mechanisms. For example, if such aggregates were to span a membrane, toxic properties may relate to the formation of pores.

gain of toxic function is likely, as constitutive or postnatal depletion of $PrP^C$ does not trigger any pathology. A lively discussion is developing on the role of abnormal $PrP^C$ topologies. Targeting of PrP to the cytosol results in rapidly lethal neurodegeneration (yet without $PrP^{Sc}$), and proteasome inhibition induces a slightly protease-resistant PrP species in cultured cells, which may be self-sustaining—at least for a while (Ma and Lindquist, 2002; Ma et al., 2002). Therefore, prion toxicity may start with retrotranslocation of $PrP^C$ from the endoplasmic reticulum to the cytosol, in conjunction with impaired proteasomal function. While PrP is clearly toxic in the cytosol, the details of how it may get there are debated. Cytosolic PrP retains its secretory leader peptide and does not contain a glycosyl phosphatidyl inositol anchor, suggesting that it never enters the endoplasmic reticulum (Drisaldi et al., 2003). Whether toxicity of cytosolic PrP is universal, however, is currently quite hotly discussed (Roucou et al., 2003). On the other hand, Lingappa found that $PrP^C$ assumes a transmembrane topology ($^{Ctm}$PrP), whose concentration correlates with neurotoxicity (Hegde et al., 1998, 1999). These data suggest that $^{Ctm}$PrP represents a major toxic moiety.

We still know nothing of the biochemical pathways leading to brain damage, be they triggered by cytoplasmic PrP or by $^{Ctm}$PrP; these may lead to the identification of therapeutic targets and may share components with other neurodegenerative diseases.

## The Future of Prion Therapeutics

An impressive wealth of molecules was touted as potential antiprion lead compounds. However, none of these therapeutical leads have proven their usefulness yet in clinical settings, and some have conspicuously failed. One of the possible problems derives from the fact that most antiprion compounds were identified in cell culture assays, where chronically prion-infected neuroblastoma cells are "cured" of their $PrP^{Sc}$ and prion burden. A startling variety of substances appears to possess such prion-curing properties; a nonexhaustive list includes compounds as diverse as Congo red (Caughey and Race, 1992), amphotericin B, anthracyclins (Tagliavini et al., 1997), sulfated polyanions (Caughey and Raymond, 1993), porphyrins (Priola et al., 2000), branched polyamines (Supattapone et al., 2001), "β sheet breakers" (Soto et al., 2000), and the spice curcumin (Caughey et al., 2003).

Disappointingly, none of these compounds proved very effective for actual therapy of sick animals—let alone patients. We therefore believe that it is premature to treat patients with alleged antiprion drugs on the sole basis of antiprion efficacy in neuroblastoma cells. This shortcut was taken in the case of quinacrine, which cures scrapie-infected cultured cells with impressive efficacy (Korth et al., 2001), yet appears to be utterly ineffective in scrapie-infected mice (Collins et al., 2002) and in CJD patients (Cooper, 2002), besides being severely hepatotoxic (Scoazec et al., 2003).

Why do scrapie-infected cells fare so poorly as a model system for prion therapy? In our experience, infection rarely hits all cells in any given culture, and the prion-infected state can be quite unstable. Therefore, one could speculate that a variety of stressors may masquerade as antiprion cures by conferring a selective advantage to noninfected cells. This interpretation would explain the puzzling observation that antiprion "cure" is brought about by compounds with no structural or biological similarities.

Cytidyl-guanyl oligodeoxynucleotides (CpG-ODN), which bind Toll-like receptor 9 (TLR9) and stimulate innate

immune responses, were reported to delay disease upon chronic administration to scrapie-infected mice (Sethi et al., 2002). The contention that immune stimulation might protect against prions is extraordinary and is difficult to reconcile with the observation that immune deficiencies of all kinds inhibit prion spread (Frigg et al., 1999; Klein et al., 1997, 1998, 2001; Prinz et al., 2003c). Besides, MyD88$^{-/-}$ mice undergo normal prion pathogenesis despite abrogation of TLR9 signaling (Prinz et al., 2003b), and we could not evidence any major effects of TLR9 stimulation on the course of disease—in a paradigm identical to that described originally (M.P., M. Heikenwälder, and A.A., unpublished data). Instead, repeated CpG-ODN administration proved extremely lymphotoxic (Heikenwalder et al., 2004)—a fact that may well explain its antiprion properties.

On a more positive note, the tremendous interest in this field has attracted researchers from various neighboring disciplines, including immunology, genetics, and pharmacology, and therefore it is to hope that rational and efficient methods for managing prion infections will be developed in the future.

***Immunotherapy against Prions?***

Prions are sturdy and their resistance against sterilization is proverbial, yet exposure in vitro to anti-PrP antisera can reduce the titer of infectious hamster brain homogenates (Gabizon et al., 1988). Anti-PrP antibodies were found to inhibit formation of protease-resistant PrP in a cell-free system (Horiuchi and Caughey, 1999). Also, antibodies (Klein et al., 2001) and F(ab) fragments to PrP (Enari et al., 2001; Peretz et al., 2001) can suppress prion replication in cultured cells.

While these data suggest the feasibility of antiprion immunoprophylaxis, the mammalian immune system is essentially tolerant to PrP$^{C}$; this is hardly a surprise, given that PrP$^{C}$ is expressed on T and B cells. Ablation of *Prnp* (Büeler et al., 1992) renders mice highly susceptible to immunization with prions (Brandner et al., 1996b), and indeed some of the best monoclonal antibodies to PrP$^{C}$ were generated in *Prnp*$^{o/o}$ mice (Prusiner et al., 1993).

Tolerance was circumvented by transgenic expression of an immunoglobulin $\mu$ chain containing the epitope-interacting region of 6H4, a high-affinity anti-PrP monoclonal antibody (Korth et al., 1997). The transgenic $\mu$ chain associated with endogenous $\kappa$ and $\lambda$ chains, some pairings lead to reactive moieties and, consequently, to high anti-PrP$^{C}$ titers in *Prnp*$^{o/o}$ and *Prnp*$^{+/+}$ mice. The buildup of anti-PrP$^{C}$ titers, however, was more sluggish in the presence of endogenous PrP$^{C}$, suggesting that clonal deletion is actually occurring. B cell clones with the highest affinity to PrP$^{C}$ are probably eliminated by tolerance, while clones with medium affinity are retained (Figure 5A). The latter sufficed to block prion pathogenesis upon intraperitoneal prion inoculation (Heppner et al., 2001). Hence, B cells are not intrinsically tolerant to PrP$^{C}$ and can, in principle, mount a protective humoral response against prions. It was then found, in a followup study, that passive transfer of anti-PrP monoclonal antibodies (in admittedly heroic amounts) can delay the onset of scrapie in mice infected with prions intraperitoneally, albeit not such infected intracerebrally (White et al., 2003).

The challenges to a practical antiprion immunization, however, are enormous. While providing an encouraging proof of principle, transgenic immunization cannot easily be reduced to practice. Further, no protection was observed if treatment was started after the onset of clinical symptoms, suggesting that passive immunization might be a good candidate for prophylaxis rather than therapy of TSEs. Active immunization, like in most antiviral vaccines, may be more effective, but is rendered exceedingly difficult by the stringent tolerance to PrP$^{C}$ (Souan et al., 2001; F. Heppner, E. Pellicioli, M.P., and A.A., unpublished results; and Figure 5B).

***Soluble Prion Antagonists***

In several paradigms, expression of two PrP$^{C}$ moieties subtly different from each other antagonizes prion replication. For example, humans heterozygous for a common *Prnp* polymorphism at codon 129 are largely protected from CJD: this effect is so important that it may have acted as selective evolutionary pressure (Mead et al., 2003). Similarly, transgenic expression of hamster PrP$^{C}$ renders *Prnp*$^{o/o}$ mice highly susceptible to hamster prions, whereas coexpression of mouse PrP$^{C}$ diminishes this effect. Transdominant single nucleotide mutations of *Prnp* have also been described (Perrier et al., 2002).

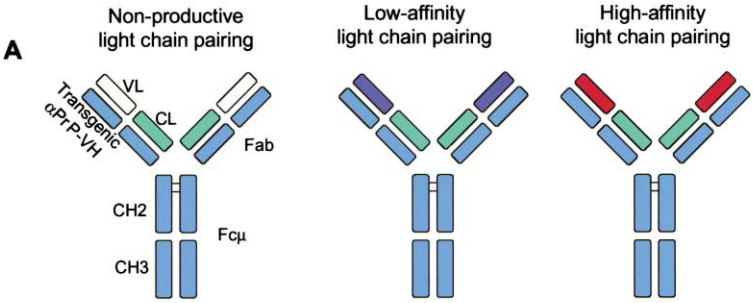
The molecular basis for these effects is unknown; perhaps the subtly modified PrP$^{C}$ acts as a decoy by binding incoming PrP$^{Sc}$ (or protein X) and sequestering it into a complex incapable of further replication.

We tested the latter hypothesis by fusing an immunoglobulin Fc$\gamma$ domain to PrP$^{C}$. The Fc$\gamma$ tail served multiple purposes: (1) ligand dimerization, which may enhance its avidity for interacting partners; (2) provision of a convenient tag for affinity purification; (3) expression of the protein as a soluble moiety, which allows for testing cell-autonomous effects; and (4) increased stability in body fluids. Excitingly, the PrP-Fc$_2$ fusion protein was found to compete with PrP$^{C}$ for PrP$^{Sc}$ (Figure 6) and to prolong the latency period of prion infection upon expression in transgenic mice (Meier et al., 2003). It will be exciting to determine whether PrP-Fc$_2$ can act cell-autonomously when delivered as a drug. If that proves true, soluble prion protein mutants may represent useful prionostatic compounds.

## Prion Diagnosis: Weaknesses and Challenges

Like in any other disease, early diagnosis would significantly advance the chances of success of any possible interventional approach. But when compared to other fields of microbiological diagnostics, the tools for prion diagnosis appear to be depressingly unsophisticated. Presymptomatic diagnosis is virtually impossible, and the earliest possible diagnosis is based on clinical signs and symptoms. Hence, prion infection is typically diagnosed after the disease has considerably progressed.

A significant advance in prion diagnostics was accomplished in 1997 by the discovery that protease-resistant PrP$^{Sc}$ can be detected in tonsillar tissue of vCJD patients (Hill et al., 1997b). It was hence proposed that tonsil biopsy may be the method of choice for diagnosis of vCJD (Hill et al., 1999). Furthermore, there have been reports of individual cases showing detection of PrP$^{Sc}$ at preclinical stages of the disease in tonsil (Schreuder et al., 1996) as well as in the appendix (Hilton et al., 1998), indicating that lymphoid tissue biopsy may be useful for




B

| Genetic backround | procedure | fate of B-lymphocytes |
|---|---|---|
| *Prnp*$^{o/o}$ | VH transgenesis | maturation |
| Wild-type | VH transgenesis | moderate deletion |
| PrP overexpressor | VH transgenesis | mainly deletion |
| *Prnp*$^{o/o}$ | immunization | vigorous response |
| Wild-type | immunization | no response |

Figure 5. Affinity of Antibodies, Tolerance, and Immunity against Prions

(A) When forced to express a transgenic heavy chain with anti-PrP specificity, B lymphocytes may couple it to a large repertoire of endogenous light chains. Some of the VH-VL pairs (variable domains of heavy and light chains) may yield very high-affinity antibodies, whereas others will have low or no affinity. (B) Mendelian crosses of α-PrP-VH transgenic mice with *Prnp*$^{o/o}$, wild-type, and PrP$^{C}$-overexpressing transgenic mice informed on tolerogenic constraints. In the absence of endogenous PrP$^{C}$, mouse sera exhibited high anti-PrP$^{C}$ titers. In wild-type mice, anti-PrP$^{C}$ titers despite some clonal deletion, whereas massive overexpression of PrP$^{C}$ led to dramatic lymphopenia (Heppner et al., 2001). Instead, active immunization yields consistently high anti-PrP$^{C}$ titers only in *Prnp*$^{o/o}$ mice. The permissivity of B lymphocytes to expression of anti-PrP$^{C}$ specificities implies that tolerance to PrP$^{C}$ is predominantly dictated by T-helper constraints. CH: Constant region of the heavy chain. Fab: antigen binding fragment. Fcμ: IgM-specific heavy chain.

diagnosing presymptomatic individuals. These observations triggered large screenings of human populations for subclinical vCJD prevalence using appendectomy and tonsillectomy specimens (Glatzel et al., 2003b). PrP$^{Sc}$-positive lymphoid tissue was long considered to be a vCJD-specific feature that would not apply to any other forms of human prion diseases (Hill et al., 1999). However, a recent survey of peripheral tissues of patients with sporadic CJD has identified PrP$^{Sc}$ in as many as one-third of skeletal muscle and spleen samples

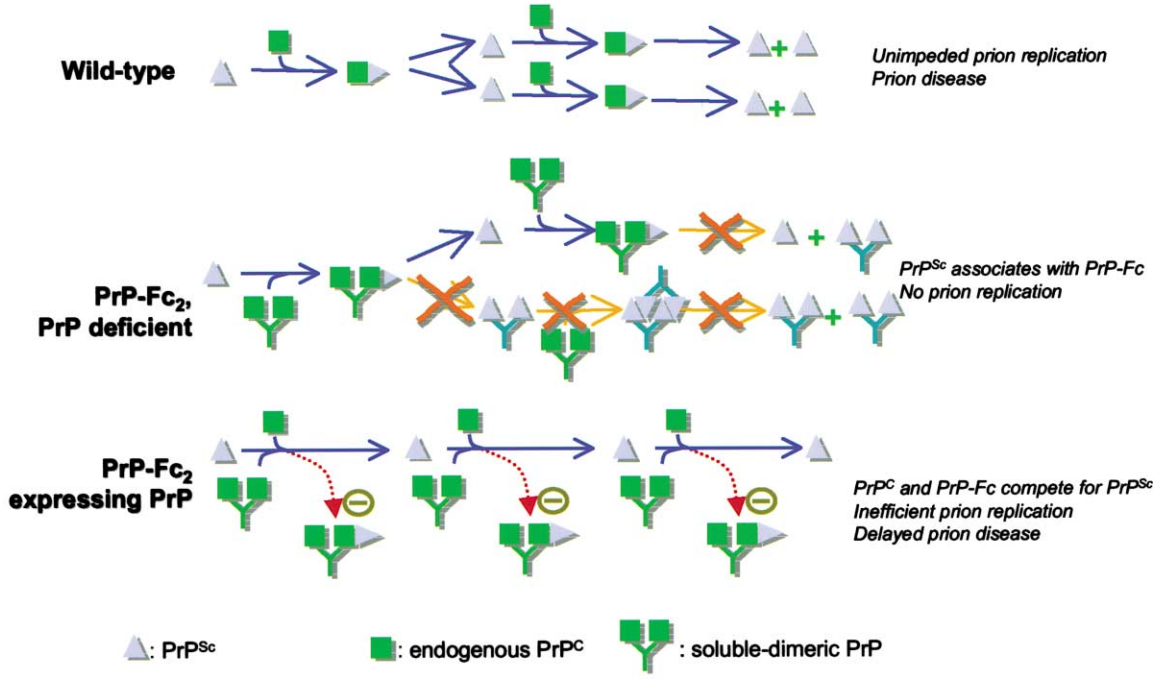



Figure 6. A Model for the Antiprion Action of PrP-Fc$_2$

The template refolding model of prion replication (top) postulates a transient dimerization of PrP$^{C}$ and PrP$^{Sc}$. As a result, PrP$^{Sc}$ would impart its own β sheet-rich, protease-resistant conformation onto PrP$^{C}$. In the absence of PrP$^{C}$, soluble dimeric PrP does not support replication of the infectious agent, nor formation of a protease-resistant moiety (middle). Although several lines of evidence indicate that it can associate with PrP$^{Sc}$, this association is nonproductive. Mice coexpressing PrP$^{C}$ and soluble dimeric PrP replicate prions and eventually develop scrapie. However, the kinetics with which scrapie pathology develops, prion infectivity replicates, and PrP$^{Sc}$ accumulates is slower than in wild-type mice. All experimental evidence presented here suggests that PrP-Fc$_2$ sequesters incoming as well as nascent PrP$^{Sc}$ and renders it unavailable for further template-directed conversion of PrP$^{C}$ (bottom).

(Glatzel et al., 2003a), as well as the olfactory epithelium of patients suffering from sCJD (Zanusso et al., 2003). These unexpected findings raise the hope that minimally invasive diagnostic procedures may take the place of brain biopsy in intravital CJD diagnostics.

The sensitivity of $PrP^{Sc}$ detection was significantly improved by the sodium phosphotungstic (NaPTA) precipitation method (Safar et al., 1998; Figure 1). By concentrating $PrP^{Sc}$ prior to Western blot analysis, this procedure improves the sensitivity of diagnostic assays by as much as 4 orders of magnitude (Wadsworth et al., 2001). An interesting development was brought about by the conformation-dependent immunoassay (CDI), in which conformational differences of PrP isoforms are mapped by quantitating the relative binding of antibodies to denatured and native protein (Safar et al., 1998). Rather than relying on protease resistance, the CDI measures a variety of misfolded PrP isoforms, which may increase its sensitivity (Bellon et al., 2003; Safar et al., 2002).

Be this as it may, all techniques described above suffer from the fact that $PrP^{Sc}$ continues to represent a surrogate marker for prion infectivity—since (1) $PrP^{Sc}$ has not been incontrovertibly shown to be congruent with the prion, and (2) several manipulations in vitro and in vivo can render $PrP^{C}$ protease resistant without bestowing infectivity on it (Jackson et al., 1999). Therefore, determination of prion infectivity by bioassay remains the golden standard; like in Pasteur's age, the concentration of the infectious agent is determined by inoculating serial dilutions of the test material into experimental animals, and the dilution at which 50% of the animals contract the disease (termed $ID_{50}$) is determined. Naturally, this system is riddled with inconveniences: scores of animals need to be sacrificed, and the incubation times are lengthy (transgenetic overexpression of $PrP^{C}$ can help, but only to some extent). Also, the method tends to be breathtakingly inaccurate: the inoculation schemes used in most studies typically suffer from standard errors of $\pm 1$ order of magnitude!

A radical improvement of this situation is likely to be brought about by the use of prion-susceptible cell lines (Bosque and Prusiner, 2000; Race et al., 1987). The determination of prion infectivity endpoints in cultures of highly susceptible cells combines the sensitivity and intrinsic biological validity of the bioassay (i.e., direct measurement of the infectivity) with the speed and convenience of an in vitro methodology amenable to medium-throughput automation (Klohn et al., 2003).

## Unresolved Problems in Prion Science

The study of prions has taken several unexpected directions over the past few years. However, the areas that are still obscure do not relate only to the details; some of them concern the core of the prion concept (Chesebro, 1998). In summary, there are five large groups of questions regarding the basic science of prion replication and of development of transmissible spongiform encephalopathies diseases:

- Which are the molecular mechanisms of prion replication? How does the disease-associated prion protein, $PrP^{Sc}$, achieve the conversion of its cellular sibling, $PrP^{C}$, into a likeness of itself? Which other proteins assist this process? Can we inhibit this process? If so, how?
- What is the essence of prion strains, which are operationally defined as variants of the infectious agent capable of retaining stable phenotypic traits upon serial passage in syngeneic hosts? The existence of strains is very well known in virology, but it was not predicted to exist in the case of an agent that propagates epigenetically.
- How do prions reach the brain after having entered the body? Which molecules and which cell types are involved in this process of neuroinvasion? Which inhibitory strategies are likely to succeed?
- The mechanisms of neurodegeneration in spongiform encephalopathies is not understood. Which are the pathogenetic cascades that are activated upon accumulation of disease-associated prion protein and ultimately lead to brain damage?
- What is the physiological function of the highly conserved, normal prion protein, $PrP^{C}$? The *Prnp* gene encoding $PrP^{C}$ was identified in 1985 (Basler et al., 1986; Oesch et al., 1985), *Prnp* knockout mice were described in 1992 (Büeler et al., 1992), and some $PrP^{C}$-interacting proteins have been identified (Oesch et al., 1990; Rieger et al., 1997; Yehiely et al., 2002; Zanata et al., 2002). Yet the function of $PrP^{C}$ remains unknown!

The questions described above deserve to be addressed with a vigorous research effort. Their study is likely to yield fundamental insights into the characteristics of these novel and essentially mysterious agents and may yield useful leads for the diagnosis and therapy of prion diseases.

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