ON THE INFECTIVITY AND PROPAGATION OF PRIONS

- WHO'S BAD? -

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CONTENTS

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ABSTRACT

The existence of proteins with an altered conformation underlie diseases such as Creutzfeldt-Jakob in human and scrapie in sheep which are collectively called Transmissible Spongiform Encephalopathies (TSEs). These conformationally altered proteins are called prions which can form aggregates/plaques and after a long incubation period in the sporadic forms of disease, results in a short disease phase and death. The nature of the infective particle has long been debated and many suggestions have been made such as a (slow) virus, virino and protein. The prion/protein-only hypothesis seems to enjoy widest acceptance as no nucleic acids have been found in isolates and the infective particle is resistant to treatments that are known to disrupt viruses and bacteria. How a protein is able to contain 'information' and 'replicate' itself is very intriguing. The mechanism(s) underlying prion propagation and infectivity have been widely investigated and many hypotheses are posed which differ in their method of conversion, the role of aggregates and dynamics. Despite much research the past decades, the process of neurodegeneration is still not understood in much detail and the toxic compound, if there is even just one, is not elucidated. This year, Sandberg et al. distinguished two phases in the disease process and suggested an uncoupling of prion propagation and infectivity, and toxicity to the cell. This uncoupling explains how high titers of prions can be found in healthy organisms whereas the opposite is also seen. Many different mechanisms can be proposed to explain this uncoupling. The involvement of other cellular factors is almost certain but in what way, is so far not clear. What becomes clear is that there is no one structure for toxic compound and there is no consensus regarding the toxicity of fibrillar and non-fibrillar oligomers and – aggregates. So far, not many humans have been affected by prion disease spread via cattle (in food), but thousands of animals have died and been sacrificed in order to contain outbreaks. In order to prevent major future outbreaks in human as well as other animals, the mechanism of infection and toxicity need to be elucidated.

ABBREVIATIONS

BSE: bovine spongiform encephalopathy
CJD: Creutzfeldt-Jakob disease (s: sporadic, v: variant)
CNS: central nervous system
DRM: detergent resistant membrane (-domains, rafts)
FFI: fatal familial insomnia
GPI anchor: glycosylphophatidyl inositol anchor
GSS: Gerstmann-Sträussler-Scheinker
IAPP: islet amyloid polypeptide
PrP: prion protein
- PrP^{C}: cellular conformation, PK sensitive, PrP-sen
- PrP^{Sc}: scrapie/pathogenic form, PK resistant form, PrP-res
- PrP*: intermediate form on pathway of conversion of PrP^{C} to PrP^{Sc}
- PrP^{L}: lethal form of PrP either on-pathway of conversion from PrP^{C} to PrP^{Sc} or formed as a by-product

PIPLC: phosphatidyl inositol-specific phospholipase C
PK: proteinase K
PM: plasma membrane
PMCA: protein misfolding cyclic amplification
RML: Rocky Mountain Laboratory (prions)
SN: seeded nucleation
TDR: template directed refolding
TSE: transmissible spongiform encephalopathy
WT: wild type

INTRODUCTION

HISTORY

There have been several outbreaks of an infectious disease which is characterized by neurodegeneration and eventually result in death. This disease, called Scrapie, had been known to affect sheep for several centuries [Collinge et al. 2008]. In the second half of the previous century, a similar disease, named 'Kuru', was prevalent amongst people endemic in the Fore region of New Guinea and is characterized by cerebellar ataxia and athetoid movements and as time progresses, affected individuals present with complete motor incapacity. Cognition however was generally not affected and the disease was always fatal within 6-12 months of onset [Gajdusek and Zigas, 1957; Gajdusek and Zigas, 1961]. The fact that this disease was so prevalent amongst the Fore led Gajdusek and Zigas to suggest a genetic component to the disease [Gajdusek and Zigas, 1961]. Affected brains showed spongiform encephalopathy which was similar to Scrapie. Scrapie was known to be an infectious disease and veterinarian Hadlow in 1959 pointed out the similarities between Kuru and scrapie [reviewed by Hadlow, 2008]. This shed new light on the nature of the disease and the link was made between Scrapie and Creutzfeldt-Jakob Disease (CJD) that up until then seemed unrelated [Norrby 2011]. Ritual endocannibalism was common among the Fore where the women and children consumed organs, the brain and other nervous tissue as men mostly ate the muscle tissue [review on Kuru: Mathews et al. 1968]. This, together with the infectious nature of the disease explained the observations of disease occurrence in specific age and sex groups as infected nervous tissue seemed to be the cause. When the Fore people ceased this ritual consumption of dead relatives, the incidence of Kuru decreased [Gajdusek 1976] which indicates that the disease indeed was infectious and not genetic which was initially proposed [Mathews 1967]. However, the observation that members of a family in Vienna presented with similar brain pathology led to the conclusion that this disease must be hereditary [reviewed by Hainfellner et al. 1995]. Since pathology in affected family members was very similar to that seen in scrapie and Kuru, which are infectious, the theory was proposed that these diseases which show such a similar pathology, might be a single disease type which somehow can be infectious as well as hereditary. The infections nature of these diseases was experimentally shown by Gajdusek et al. in 1966 by demonstrating transmission of Kuru to chimpanzees and Gibbs et al. in 1980 who demonstrated that Kuru, Creutzfeldt-Jakob Disease (CJD) and Scrapie indeed were infectious and that they could be transmitted via the oral route to monkeys.

NATURE OF THE INFECTIOUS AGENT

There have been several hypotheses proposed regarding the nature of the disease. These include the conventional virus view in which a virus is causing the disease(s) [Rohwer 1984]; the virino concept where it is thought that TSE (Transmissible Spongiform Encephalopathy) pathogens are low molecular weight nucleic acid molecules (supposedly small RNAs) which replicate and are transmitted in a complex together with a host specific protein [Hope 1994]. This mechanism was proposed to explain the lack of a specific humoral or celular immune response upon prion infection [Porter et al. 1973; Kingsbury et al. 1981] and also to explain the strain variation [Kimberlin 1982] (strains are explained later on). Studies to identify the pathogenic protein in the amyloid plaques that were seen in brains of infected animals and human have shown that it is a host-encoded protein, PrP [discussed by Aguzzi and Calella, 2009]. Also, a unified theory has been proposed by Weissmann in 1991 which states that the conversion of PrP^{C} (the cellular form of the prion protein PrP) to an abnormal conformer that results in disease is the essential pathogenic event. But, a small host-specified nucleic acid (designated the co-prion) associates with PrP^{Sc} (the pathologic conformation of PrP) which is a crucial component that modulates strain specificity. However, nowadays the prion/protein-only hypothesis seems to best explain experimental data [Soto 2011]. The infectious agent is thought to consist solely of protein; it is a conformational isoform of the host encoded protein PrP^{C}. The native protein is sensitive to degredation by proteinase K (PK) and therefore often named PrPsen, whereas the pathogenic form is partially resistant to PK digestion [Prusiner 1982], denoted PrPres. This difference in sensitivity is used in assays to detect the resistant form which is designated $PrP^{27\text{-}30}$ since the fragment size after PK digestion is 27 to 30 kDa [McKinley et al. 1983]. PK can partially digest PrP^{Sc} at residue 97 or 82 resulting in large fragments (27-30 kDa) as well as smaller fragments, such as of size 21 and 19 kDa respectively of which these smaller fragments are unglycosylated [discussed by Jansen, Chapter two of his thesis]. The fact that a misfolded protein is causing disease explains how they can be infectious, hereditary as well as sporadic. Many observations support this hypothesis (Table 1).

Table 1: Evidence pro/contra the protein-only hypothesis

Pro:	**Reference(s):**
- Infectious PrP^{Sc} found in brain isolates	- Review Aguzzi and Calella, 2009
- No nucleic acid found	- Prusiner 1982
- Prnp knock-out mice are resistant to infection and can not propagate the infective agent	- Büeler et al. 1993
- Mice with mutant Prnp develop spontaneous prion disease	- Hsiao 1990 et al. 1990
- The infectious agent is resistant to treatments that kill bacteria and viruses	- Alper 1966
- All familiar cases are linked to mutations in the PRNP gene and some mutations predispose carriers to sporadic disease	- Mead 2006, Collinge 2001
- Synthetic prions generated using truncated recombinant mouse PrP are infectious	- Legname et al. 2004
- $PrP^{0/0}$ mice with $PrP^{+/+}$ mouse brain graft show pathology after intracerebral injection but only in the graft	- Aguzzi et al. 1998
- Protein Misfolding Cyclic Amplification (PMCA): the in-vitro chemical conversion of the PK sensitive to the PK resistant form of the prion protein	- Bieschke et al. 2004
- Hamster prions can only infect transgenic mice containing the hamster Prnp gene, isolates from these mice can infect hamsters	- Scott et al. 1989
- The species barrier can be explained by differences in the Prnp gene = slightly different conformation	- Collinge et al. 1999
- Infectivity is reduced by agents that destroy protein structure and by αPrP antibodies	- Gabizon et al. 1988
Contra:	**Reference(s):**
- Many strains exist which differ in their incubation time and neuropathology, how can this be explained when one protein is involved? (this is explained by the many slightly alternate conformations PrP^{Sc} can have as well as structure of the infectious seed)	- Somerville 2002 (Wadsworth et al. 1999; Lansbury and Caughey, 1995)
- *In vitro* conversion of PrP^{C} to PrPsc required cell lysates, solely purified protein was not sufficient (however, the necessary co-factor(s) does not need to be a nucleic acid)	- Saborio et al. 1999
- PMCA: infectivity of the newly produced PK resistant form of PrP did not increase as much as the conversion rate of cellular to PK resistant form which suggests other factors necessary in the process of production of infective species	- Bieschke et al. 2004

Prion disease such as Scrapie in sheep and Kuru in humans are thus considered to result from misfolded PrP^{C} (the cellular form of the prion protein PrP), other diseases like these are Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS), the recently described protease-sensitive prionopathy [Jansen et al. 2010;

Gambetti et al. 2008] as well as the very rare Fatal Familial Insomnia (FFI) [Medori et al. 1992]. Animals (mice, sheep, goat, monkeys) as well as cell-cultures are used to study prion disease [Baron 2002b; Arellano-Anaya et al. 2011]. Yeast and fungi also possess prions, but as these organisms are so different from mammals, are used only for study of protein aggregation/amyloid formation [Sigurdson et al. 2005; Summers and Cyr 2011; Tuite et al. 2011]. Study of yeast can give more insight into basal processes whereas studies on cell culture and whole animals are needed to uncover the process of neurodegeneration.

Prion diseases are not alone in that they result from the misfolding of proteins. Alzheimer's disease (involving Aβ), as well as Parkinsonism with Lewy bodies disease (α synuclein) and type II diabetes (islet amyloid polypeptide (IAPP) in the pancreas) are associated with accumulation/aggregation of misfolded proteins [Petkova et al. 2005; Yonetani et al. 2009; Yagui et al. 1995] which lead to neurodegeneration [reviewed by Caughey and Lansbury, 2003] and in the case of IAPP, to β cell death in the pancreas [Dupuis et al. 2011]. These proteins thus fit in the same biochemical class as prions [Frost and Diamond, 2010] and since many patients suffer from these afflictions, the importance of understanding prion disease progression and toxicity are clear. Since in prion disease, a misfolded form of the native protein PrP^{C} is found, I will give an overview of its properties and function. The misfolded form has long been considered to be responsible for infectivity as well as toxicity. Recent findings indicate however, an uncoupling of the two [Sandberg et al. 2011]. After introducing the topic, I will discuss findings by Sandberg et al. and mechanisms of prion propagation (multiplication). Subsequently, I discuss proposed mechanisms of prion propagation and will end by proposing possible mechanisms of this uncoupling of infectivity and toxicity in light of earlier proposed mechanisms. An uncoupling of infectivity and toxicity will change our understanding of prion diseases which, eventually, will lead to identification of new therapeutic targets.

PrP^{C}

PrP^{C} (the cellular form of PrP) is encoded by the *PRNP* gene (in humans on the short arm of chromosome 20) and the entire open reading frame (ORF) is contained in one exon [Basler et al. 1986] which excludes alternative splicing as a cause for the existence of PrP^{Sc} (the Scrapie form of PrP). The protein is synthesized as a pre-pro-PrP of 253 amino acids. The leader peptide sequence of residues 1-22 at the N-terminus as well as the GPI anchor peptide signal sequence of residues 232-253 are removed in the ER [discussed by Li et al. 2010]. PrP's N-terminus is flexible whereas the C-terminus contains a globular domain with two β-sheets and

three α-helices [Donne et al. 1997]. PrP^C is 33 to 35 kD [Oesch et al. 1985; Basler et al. 1986] and gets its Asn-linked oligosaccharides modified and sialylated in the Golgi apparatus (N-linked glycosylation) [Endo et al. 1989] (Fig. 1).

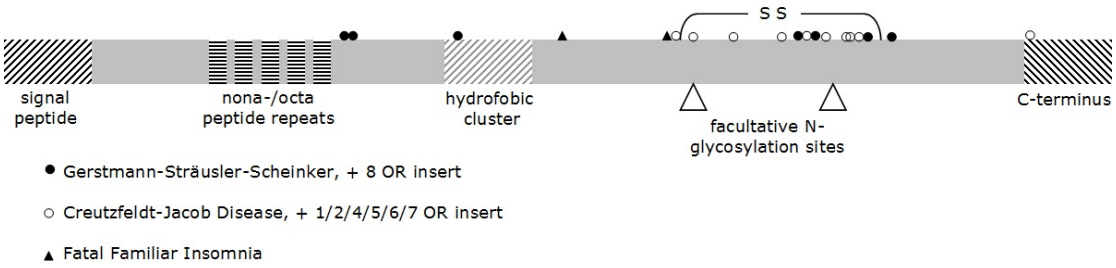


Figure 1: Schematic representation of the PRNP gene with its most important regions. Mutations associated with three disease types are indicated. Also, octarepeat (OR) insertions are found. Figure adapted from Jansen et al. 2011 (Thesis) and mutations: http://www.federationofscientists.org/PMPanels/TSE/Priprogene.asp

Via vesicles, PrP^C is transported to the plasma membrane (PM) after which it cycles between the cell surface (attached via its glycosyl-phophatidyl inositol (GPI) anchor) and endocytic compartments in the cell [Taraboulos et al. 1992]. PrP^C can spontaneously be released from the cell or by cleavage with phosphatidyl inositol-specific phospholipase C (PIPLC) [Zhao et al. 2006] (Fig. 2).

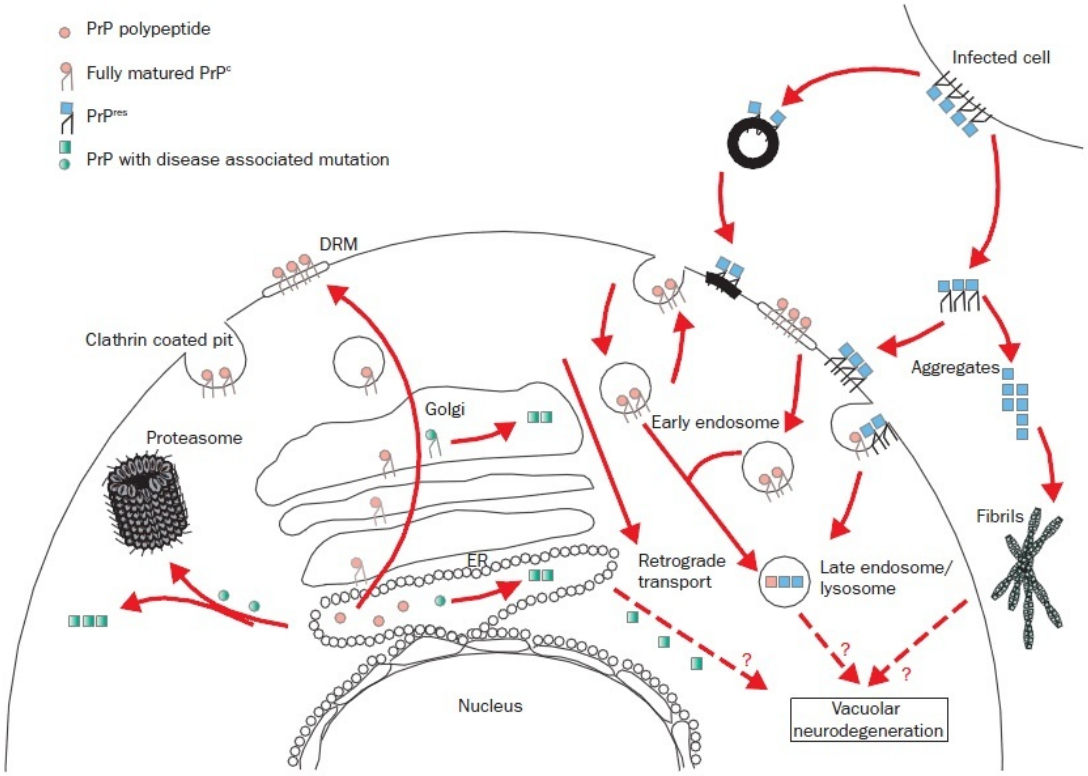


*Figure 2: PrP^C (pink circles) synthesis and cell turnover schematically shown as well as possible sites of conversion to PrPres (blue squares). Mechanisms of toxicity and subsequent neurodegeneration are not understood. * Figure and*

legend copied from Collins et al. 2004

In the PM, PrP localizes in cholesterol-rich, detergent resistant membrane (DRM) domains (also called lipid rafts) [Naslavsky et al. 1997] and can be present in the PrP^{C} or PrP^{Sc} isoform. The intactness of lipid rafts is thought to be necessary for conversion of PrP^{C} into the pathogenic form [Goold et al. 2011]. The role of GPI anchoring for disease progress is ambiguous: Chesebro et al. in 2005 showed that anchor-less mice showed infectious amyloid disease without clinical Scrapie despite neuropathological lesions but later it became clear that these mice did develop disease albeit later than controls [Chesebro et al. 2010; Klingeborn et al. 2011]. This is in line with findings by Jansen et al. 2010 who studied patients with a gene insertion resulting in a premature stop codon preventing GPI anchorage. Prion protein deposits as well as Tau protein accumulation (often found in Alzheimer's Disease [review Lee et al. 2011]) were accompanied by gliosis and neuronal loss.

PrP^{C} is expressed in embryonic as well as adult rodent as well as human organs and PrP gene expression is found mostly in neuronal cells but also in other tissues [Bendheim et al. 1991, Makrinou et al. 2002]. In brain, there are certain regions where after disease, PrP^{C} has been found but not PrP^{Sc}, whereas other regions showed the opposite. It is suggested by Prusiner [Chapter 153, 'The metabolic and molecular bases of inherited disease', 1995] that prions are transported along axons which, to the author, is in agreement with findings that scrapie infectivity migrated in a pattern consistent with retrograde transport [Bartz et al. 2002].

PrP^{C} is thus a protein present in many different cell types, but the physiological function of PrP^{C} is unknown. Chickens have a similar protein which is implicated in acetylcholine receptor-induction [Harris et al. 1991] which might also be the case in mammals. As young Prnp null mice show no serious developmental and growth defects, PrP^{C} seems to be redundant [Büeler et al. 1992, 1993]. Brown and Harris, in 2003, suggested a role for PrP^{C} in cellular uptake and/or efflux of copper and perhaps also zinc. This is based on observations that the nona/octapeptide repeat regions (of which PrP^{C} has one and four, respectively) can bind copper and it is shown that zinc stimulated endocytosis of PrP^{C} [Pauly and Harris, 1998]. The influence of this repeat region is illustrated by observation that Purkinje cells were displaced in the cerebellum of a patient with insertions in this region [Jansen et al. 2009]. Also, a neuro-protective function has been attributed to PrP^{C} as it is involved in cellular signaling and stimulates neurite outgrowth as well as promoted neuronal survival [Novitskaya et al. 2006; Chen et al. 2003; Kanaani et al. 2005]. The protective effects of PrP^{C} observed after ischemic events are suggested to be mediated by PrP^{C} through

activation of anti-apoptotic and anti-oxidant pathways [Spudich et al. 2005; White et al. 1999]. Interestingly, PrP is recently linked to several pluripotency genes and a crucial role for PrP is found in regulating self-renewal/differentiation status of embryonic stem cells [Miranda et al. 2011] which implies an important role for PrP in embryogenesis. What this exact role is remains to be elucidated.

Conversion of PrP^{C} to PrP^{Sc} takes place shortly after exposure of neuroblastoma cells to prions (order of minutes). This conversion takes place at the cell surface after which PrP^{Sc} was endocytosed and trafficked. Less PrP^{Sc} was generated after release of PrP^{C} by PIPLC and many cells bound/internalized PrP^{Sc} from the inoculum upon prion exposure. Some of these showed conversion of PrP^{C} to PrP^{Sc}. The authors, Goold et al. 2011, suggest that there must be other factors that affect prion conversion which was also shown by *in vitro* prion conversion which required cell lysates [Saborio et al. 1999]. The positioning of PrP^{C} in rafts is important for conversion [Taylor and Hooper, 2006; reviewed by Campana et al. 2005] since disruption of rafts resulting in abrogation of prion conversion [Goold et al. 2011]. There are likely proteins or other molecules that reside in/near rafts that aid in conversion or the raft provides a certain structural environment which favors conversion.

AGGREGATION

PrP^{C} and PrP^{Sc} differ in their conformation: PrP^{C} contains to a large extent α-helices (42 %) and little β-sheet (3 %) whereas PrP^{Sc} consists of 30 % α-helix content and 43% β-sheet [Pan et al. 1993]. This conformational change provides PrP with different properties such as the possibility to form fibrillar amyloid which are un-branched small molecules that aggregate in a uniform manner to form a stable conformation, as well as the possibility to form small diffuse deposits of non-amyloid/non-fibrillar aggregated protein [Ghetti et al. 1996; Piccardo et al. 1998]. The formation of aggregates can be reversible [Wetzel 2006] but the equilibrium may be towards aggregate formation. The β-strand direction in amyloid is perpendicular to the fibril axis [Jenkins and Pickersgill, 2001] and in prions, the β-sheets are ordered parallel to each other [discussed in Herczenik and Gebbink, 2008]. Recent findings however show that there are no universal tertiary and quaternary structures for amyloid fibrils [Meredith 2005]. It is likely that different structures will have different properties and can be the cause for the heterogeneity seen in disease.

Aggregates are found outside of the cell [review by Caughey and Lansbury, 2003] but PrP^{Sc} is also found deposited in cytoplasmic vesicles which appear to be secondary lysosomes [Taraboulos et al. 1990; McKinley et al. 1991].

SPECIES BARRIER

There is a species barrier for transmission of prion diseases: transmission between species is far less efficient than within species [Kimerlin and Walker, 1979; Taylor et al. 1986]. It was thought that this barrier could be attributed to differences in PrP primary structure between the donor and recipient [Collinge and Clarke, 2007; Prusiner et al. 1990b; Scott et al. 1993]. This is demonstrated by the observation that classical CJD prions hardly transmit to mice, but transgenic mice that express the human, and not the mouse PrP, lack a species barrier [Hill et al. 1997; Collinge et al. 1995]. However, vCJD prions, which have an identical PrP primary structure as classical CJD prions, transmit better to wild-type mice than mice expressing the human PrP [Hill et al. 1997].

STRAINS

Strains are varieties of prion disease that differ in incubation times and the distribution of neuronal vacuolation which correlates to the patterns of PrP^{Sc} deposition [review by Prusiner, 1998]. Strains have also been characterized by the protease cleavage sites of PrP-res [Bessen and Marsh, 1994; Bessen et al. 1995; Telling et al. 1996] which indicates that different conformations separate strains from each other. Chiti and Dobson in 2006 suggested that there might be slightly different conformations of subunits in aggregates and "this lack of a single unique structure, coupled with the extremely high degree of repetitive order within individual fibrils, may be the origin of the strain phenomena observed in both yeast and mammalian prions." Collinge and Clarke (in 2007) explain properties of prion strains which is nicely illustrated in figure 3.

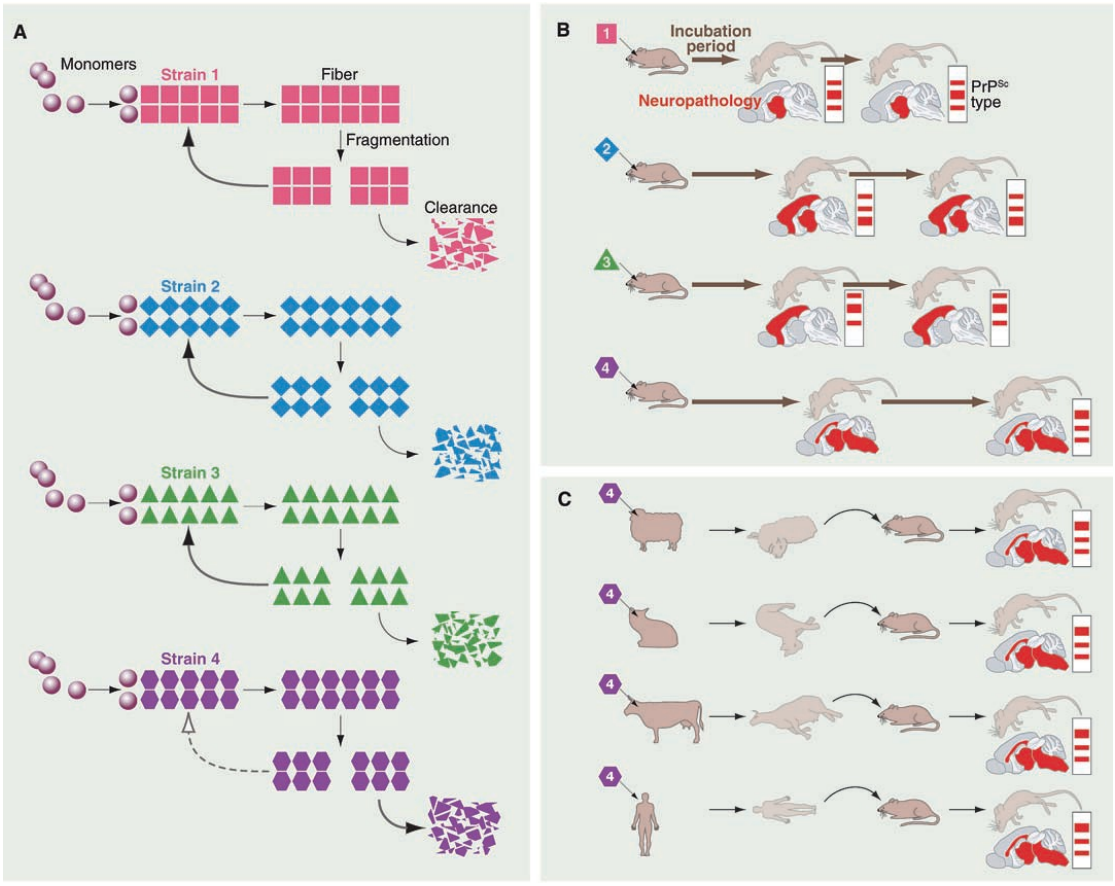


*Figure 3: Propagation of prion strains. (A) Prion propagation proceeds by recruitment of PrP monomers onto a preexisting PrP polymer template followed by fission to generate more templates in an autocatalytic manner. Distinct PrP polymer types can propagate, accounting for different strains. (B) Strains can be differentiated by characteristic incubation periods (length of arrow) and neuropathology (shaded brain area) when inoculated into defined inbred mice. Strain-specific PrP^{Sc} fragment patterns following proteolysis are illustrated in diagrammatic Western blots (vertical bars). Both biological and biochemical strain characteristics are closely maintained on serial passage in the same host expressing the same PrP^{C}. (C) Properties of a single strain may be retained after passage in a range of different species with distinct PrP^{C} sequences, when re-isolated in the original host. *Figure and legend copied from Collinge and Clarke 2007*

Collinge in 1999 proposed the 'conformational selection model'. This model states that the host PrP^{C} primary structure influences which of the possible PrP^{Sc} types are thermodynamically preferred with respect to conformation and kinetically selected during propagation. The transmission barrier is determined by the degree of overlap between the subset of PrP^{Sc} conformations allowed or preferred by PrP^{C} in the host species and the donor species. It is concluded by Collinge and Clarke in 2007 that strains and transmission barriers can thus be considered opposite sides of the same coin.

The influence of minor alterations in PrP primary structure is illustrated by the fact that in humans, the polymorphism at codon 129 determines whether BSE (bovine spongiform encephalopathy),

'mad-cow disease', can infect the individual or not. Methionine (M) or Valine (V) can be present either homozygous or heterozygous. It is suggested that homogeneous peptide amyloid is more stable that heterogeneous amyloid which was tested by Come and Lansbury in 1994 in light of the Nucleation Dependent Polymerization (NPD) model (details follow). The authors conclude that heterogeneous mixtures nucleate slower and show more soluble fibrils. This can explain the protection that heterozygotes have from prion disease since all cases of vCJD that occurred up to 2007, were in individuals with the MM phenotype [Jones et al. 2007] but an individual with the MV type showed asymptomatic vCJD upon blood transfusion from an individual who later on died of vCJD [Peden et al. 2004]. Recently, prions have been found in appendixes of Valine homozygotes [Ironside et al. 2006] which were thought to be protected from vCJD. There is a fear of a large increase in vCJD cases in the future in heterozygotes where incubation time might be longer than in homozygotes and the possible existence of chronic subclinical infection in animals (and humans) stresses importance of understanding prion diseases [Collins et al. 2004].

PROPAGATION

What is/are the mechanism(s) behind the propagation of prions? How can a misfolded protein impose its structure on a correctly folded protein? Chaperones are known to aid proteins in folding into their native conformation but can also prevent aggregation of proteins by their binding [reviewed by Wickner et al. 1999]. 'Protein X' has been proposed to represent a chaperone involved in the conversion of PrP^{C} to PrP^{Sc}, but no such protein has so far been found. It is also possible that this chaperone is not one protein but some other constituent of the PM as studies have shown that native PrP^{C} is not sufficient for in vitro prion propagation but that other factors, such as co-purified lipids, are needed [Deleault et al. 2007]. Conversion is shown to take place at the plasma membrane in neuroblastoma cell lines [Goold et al. 2011] in rafts [reviewed in Taylor and Hooper, 2006; Campana et al. 2005]. Edenhofer et al. 1996 proposed a role for the chaperone Hsp60 in prion formation as 'protein X', since this protein interacts with PrP^{C} and mutations in the regions of interaction in PrP are found in inherited cases of prion disease [Prusiner and Hsiao, 1994] but its involvement has not been studied in great detail.

INFECTIVITY

How are animals infected with prions? After oral inoculation, prion protein is first found in Peyer patches which are part of the gut-associated lymphoid tissue [Kimerlin and Walker, 1989; Beekes and McBride 2000; Heggebo et al. 2000]. Prions then spread/travel to the brain stem (especially parts of the nervus vagus) and via these

structures, reach other parts of the brain (details discussed in Tyler 2004) (Fig. 4). The importance of the lymphoid system upon peripheral prion infection is illustrated by the fact that SCID mice (deficient in mature B- and T lymphocytes) showed decreased uptake of PrP^{Sc} into the intestine [Uraki et al. 2011], however, B-cell deficient mice were not protected from intra-cerebral prion infection [Klein and Aguzzi, 2000]. It is shown that patients suffering from BSE have a fairly active immune system which implies the involvement of the immune system in establishment of infection.This is supported by the finding that knock-out of the receptor CD21/35 in mice resulted in lower attack rates of prion infection and a prolonged disease course which to the authors suggested that CD21/35, which together with PrP^{C}, is highly expressed on follicular dendritic cells, must target prions to these cells which aids in neuroinvasion [Zabel et al. 2007]. B cell deficient mice showed to be protected from peripheral prion infection and it is suggested that perhaps these cells are responsible for transport of prions to nervous tissue and/or the binding of PrP^{Sc} to antibodies might facilitate nucleation (thought to be important for infection/ propagation) [Brandner et al. 1999].

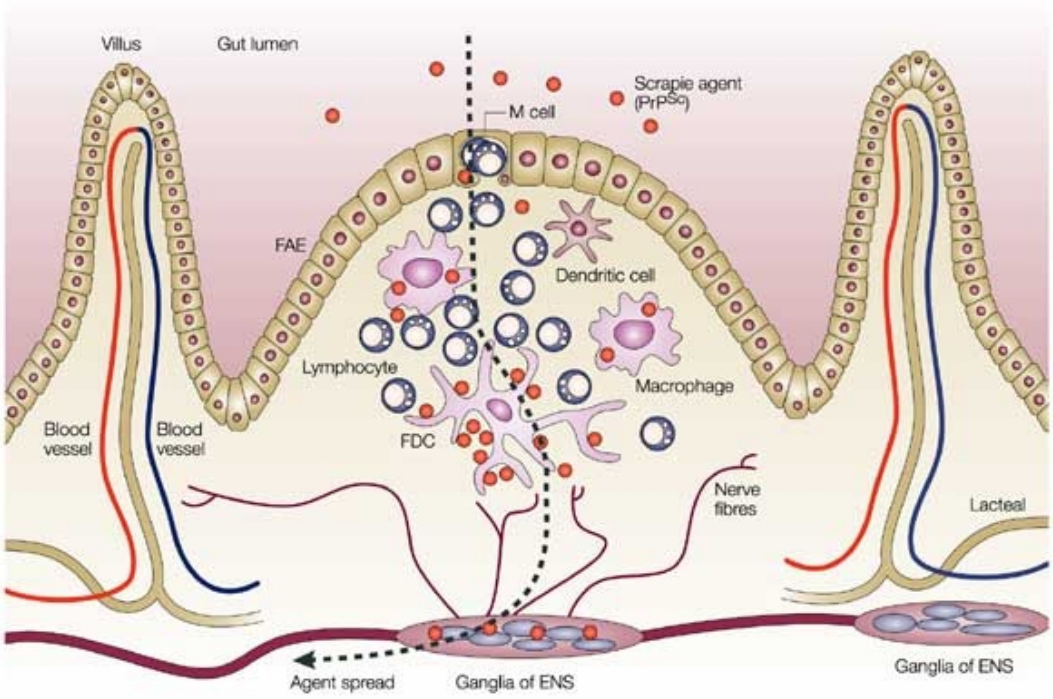


*Figure 4: Soon after ingestion, the abnormal prion isoform (PrP^{Sc}) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system. FAE, follicle-associated epithelium. *Figure and legend copied from Cashman and Caughey 2004*

Prion diseases can also transmit via contaminated surgical instruments, brain grafts or pituitary hormones [reviewed by Will, 2003]. Infection upon receiving a blood-transfusion has also occurred [Llewelyn et al. 2004] and indeed PrP^{C} as well as PrP^{Sc} are shown to circulate in the blood, cerebrospinal fluid [Volkel et al. 2001; Picard-Hagen et al. 2006]. And are able to pass the blood-brain barrier in mice [Banks et al. 2004].
How does disease spread from one cell to another and in this way, infect large parts of the nervous tissue? It is conceivable that misfolded proteins, which reside outside of the cell, can, upon interaction with correctly folded PrP^{C} from another cell, impose a conformational change or, alternatively, PrP^{Sc} 'travels' from an infected cell to a naïve cell where upon encounter with PrP^{C}, the latter is converted to PrP^{Sc} [Brandner et al. 1996]. However, recent results indicate the involvement of multivesicular bodies (MVBs) and their exosomes (often also referred to as microsomes) in neuro-degenerative diseases [reviewed in Von Bartheld and Altick, 2011]. MVBs are endosomal organelles consisting of multiple vesicles enclosed in a single layer outer membrane. Many proteins are found in these vesicles, amongst which also prions [reviewed in Von Bartheld and Altick, 2011]. They are known to be involved in many endocytic- and trafficking functions such as protein sorting, transport, storage, release and recycling and they release exosomes [reviewed in Keller et al. 2006]. PrP^{C} and PrP^{Sc} have been found in MVBs and can be excreted via these exosomes [reviewed in Von Bartheld and Altick, 2011]. The number of MVBs is increased in prion-infected animals, there were enlarged and show aberrant morphology in glia [Ersdal et al. 2009]. What the exact role of exosomes is in prion disease, is not established but exosome excretion might be necessary for infection of nearby cells.

NEURODEGENERATION-TOXICITY

A big question that still remains unanswered is what the cause is for neurodegeneration in prion diseases. It has been suggested that depletion of PrP^{C} causes neurodegeneration [Radford and Mallucci, 2009]. As Prnp null mice develop and grow, this seems not the case [Büeler et al. 1992, 1993]. However, these mice were very sensitive to ischemic or traumatic brain damage which resulted in more severe apoptotic neuronal cell death compared to wild-type mice [McLennan et al. 2004; Sakurai-Yamashita et al. 2005; Weise et al. 2004; Hoshino et al. 2003]. The necessity for PrP^{C} expression in neurodegeneration is illustrated by the fact that Prnp knock-out mice are resistant to prion infection [reviewed by Weissmann and Fechsig, 2003] and depletion of PrP from infected human as well as mouse cells (neuronal and fibroblast) with siRNA for PrP abolished toxic effects [Novitskaya 2006]. RNAi treatment of PrP of infected mice resulted in prolonged life, prevented onset of behavioral

deficits, reduced spongiform degeneration and protected against neuronal loss [White et al. 2008]. It is clear that PrP^{Sc} is involved in disease but there are people who question direct involvement of PrP^{Sc} in toxicity. This is based on the fact that subclinical prion infection might exist which differ from pre-clinical forms: animals propagate and accumulate infectious prions (high levels) but live a normal lifespan and do not display disease symptoms [Frigg et al. 1999]. Collinge and Clarke 2007 elaborate on this and summarize results that argue direct involvement of PrP^{Sc} in neurotoxicity: the occurrence of prion diseases that coincide with very low PrP^{Sc} brain levels [Collinge et al. 1995b; Hsiao et al. 1999; Medori et al. 1992b]; the fact PrP^{Sc} deposit distribution does not necessarily correlate to clinical signs and the fact that PrP^{Sc} is not directly toxic to neurons that do not express PrP^{C} [Büeler et al. 1993] as well as the fact that knock-out of neuronal PrP^{C} expression during infection protected mice from disease [Mallucci et al. 20037]. This is further supported by the fact that $Prnp^{+/0}$ mice after Rocky Mountain Laboratory (RML) prion infection show high levels or PrP^{Sc} (similar to wild type mice) but show a much longer incubation period. Also, mice over-expressing PrP^{C} (tg20, stated about 10 fold WT expression level) die early after RML prion infection with low PrP^{Sc} levels at the end stage of disease [Fischer et al. 1996].

As aggregation of misfolded protein has been implicated in neuronal cell death [Chiti and Dobson, 2006], these structures could be toxic, but amyloid is not found in all cases of prion disease which questions this relationship [Watanabe and Duchen, 1993]. Protein folding diseases show amyloid fibrils/deposits of α-synuclein, Aβ peptide and prion protein but also formation of non-fibrillar soluble oligomers has been observed [discussed by Novitskaya et al. 2006]. These soluble oligomers were either prefibrillar intermediates that were formed on-pathway to the mature amyloid [Kirkitadze et al. 2001; Kaylor et al. 2005; Zhu et al. 2004; Bitan et al. 2003] or as off-pathway products via alternative aggregation mechanisms [Bocharova et al. 2005; Baskakov et al. 2002; Souillac et al. 2002]. Non-fibrillar oligomers are intrinsically toxic to cells, "even when they are formed from proteins that are not related to any known conformational disease" [Bucciantini et al. 2002, 2004]. Novitskaya et al. 2006 showed that not only soluble oligomers of misfolded protein but also mature amyloid fibrils are toxic to cultured neuroblastoma, fibroblasts and primary neuronal cells.
This together with co-morbidity that is found in patients (the existence of different aggregates made of different protein [personal communication with Prof. Dr. Rozemuller] suggests that oligomeric species share a common mechanism of cytotoxicity [Kayed et al. 2004; Demuro et al 2005]. The formation of aggregates would then protect cells from the cytotoxic effects of soluble oligomers

[Bucciantini et al. 2002; Caughey and Lansbury, 2003; Kirkitadze et al. 2002].

Prions are thus very interesting proteins in that misfolded conformations result in neurodegenerative diseases. The existence of many differences in pathology and incubation time between different strains is fascinating as well as effects of Prnp knock-out/down on disease. How prions impose their conformation on the native cellular protein and in what way this is toxic, remains to be elucidated. Recent findings which uncouple infectivity from toxicity will be discussed and mechanisms of propagation explained. The recent findings will critically be looked at and suggestions made for further experiments as well as speculations on the mechanism of production of toxic species. The uncoupling of infectivity and toxicity forces us to look at prions differently: not as a homogeneous group of misfolded protein that is toxic, but as a group of conformationally different oligomers/polymers with different properties and roles in prion disease.

SANDBERG et al. 2011: Prion propagation and toxicity *in vivo* occur in two distinct mechanistic phases

First we recapitulate findings of Sandberg et al. 2011. Effects of inoculation of mice with RML (Rocky Mountain Laboratory) mouse prions were studied. Four different mouse strains were used:

Mouse	*PrP^C expression level*
$Prnp^{0/0}$	No expression
$Prnp^{+/+}$	Wild-type
$Prnp^{+/0}$	50% of wild type (WT)
Tg20	8-fold WT

After infection, mice were killed at defined time-points or at onset of clinical disease (Fig. 5a). Infectivity titres (the number of infectious units) were recorded at several time points after infection. Results showed the existence of two distinct phases of prion propagation during the incubation period (period from infection to death) of RML prion infection in the mice tested: phase 1 showed exponential propagation of prions (infectious units) until a clearly defined limit is reached (Fig. 5b) which is similar for the tg20 and PrnpP+/+ mice, in hemizygous mice, it takes longer. When the number of infectious units reaches a plateau, phase 2 starts. During this phase, infectivity does not increase anymore and onset of clinical disease takes place at the end of phase 2 (Fig. 5b). The length of phase 2 was found to be inversely proportional to PrP^C expression level (Fig. 5c) and this level was speculated to be directly proportional to the rate of formation of the toxic species which are thought to be different from the infectious species. As prion titres (infectious units) did not increase after a certain level was reached, which was similar for all except the null mice, a limiting factor must exist which is not PrP^C expression. However, how rapid toxic species form was dependent on PrP^C expression. This clearly shows an uncoupling of infectivity and toxicity.

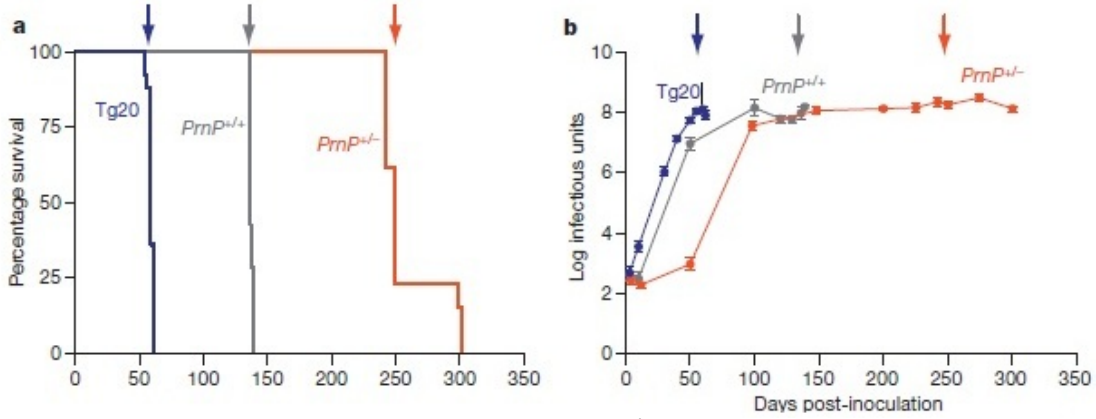


Figure 5 a/b: a: survival curves in mice. $Prnp^{0/0}$ mice were not included as they do not develop disease. Mean incubation periods (infection-onset clinical disease)

are indicated by arrows. B: Log tissue culture infectious units per gram brain, bars: sem. Group size 3-6, arrow: onset of clinical disease.

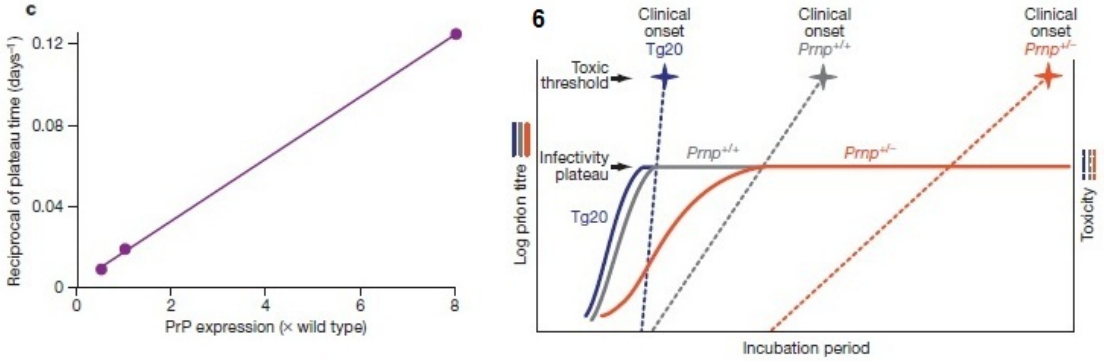


Figure 5 c: The reciprocal of the plateau time in days plotted against the expression level or *PrP^C in the mice. The length of phase 2 is inversely proportional to PrPc expression level.*
Figure 6: Kinetics of prion propagation and toxicity. The solid lines represent prion replication which is exponential until a limiting titre of prions is reached (phase 1). This is independent of PrPc concentration. A pathway switch occurs leading to the production of toxic species (dotted lines) at a rate linearly dependent on PrPc concentration (phase 2). Toxic species do not accumulate until infectivity saturates and clinical signs occur after toxic threshold is crossed.

The authors explain their findings of an uncoupling of prion infective titer and neurotoxicity by the model proposed by the same group in the past years [Hill and Collinge, 2003; Collinge and Clarke, 2007]. In this mechanism, oligomeric structures denoted 'PrP^L' (lethal) made from PrP^C are neurotoxic. PrP^{Sc} particles (which in this model are 'just' infectious) act as a catalytic surface for the production of PrP^L. Production of PrP^L was directly proportional to cellular PrP^C concentration and this determined the time to onset of clinical disease. Production of PrP^L was initially proposed to happen during 'production' of PrP^{Sc} [Hill and Collinge, 2003; Collinge and Clarke, 2007], but in light of these recent findings, Sandberg and colleagues believe PrP^L production to start at the transition from phase 1 to phase 2, so when infectivity has reached a plateau.

The authors summarize that propagation of prion infectivity proceeds in a single phase (1) of exponential autocatalytic conversion. PrP^C addition is rate-limiting only at low expression levels (as seen in the hemizygous mice). The authors state that the rate-limiting step at high concentrations of PrP^C must be either a structural rearrangement following PrP^C – PrP^{Sc} interaction [Collinge and Clarke, 2007] or a step in the division of the extended PrP^{Sc}. In phase 2 however, there is no increase in infectivity and the rate of formation of the toxic species is directly proportional to PrP^C concentration. The nature of conversion has changed as it must be rate-limited by the addition of PrP^C. The authors state "That there are closely similar levels of infectivity at the end of phases 1 and 2, irrespective of PrP^C expression level, indicates that there is either a key cofactor, at effectively fixed concentration, or a saturable

number of replication sites [Dickinson et al. 1972], that limits prion production." The authors refer back to their publication from 2007 [Collinge and Clarke] where they proposed that the neurotoxic species are oligomeric forms of PrP (denoted PrP^{L}) which are not on-pathway for prion propagation but produced in a separate, but linked pathway where PrP^{Sc} particles act as a catalytic surface for the production of PrP^{L}.

In order to understand how this uncoupling of infectivity and toxicity might work, we need to look at mechanisms proposed for the propagation of prions.

DISCUSSION

PROPAGATION HYPOTHESES

The most widely accepted explanation of the infectious agent in prion disease is the prion/protein-only hypothesis proposed by Prusiner in 1982. TSE are caused by self-replicating proteins (called prions) and are identified as proteinase-K resistant structural isoforms (PrP-res/ PrP^{Sc}) of the cellular protein PrP^{C} [Prusiner 1982, Science]. But how are prions propagated? Two hypotheses have so far dominated the field: template directed refolding/templated conformational change/conformational model (referred to as TDR), and the seeded nucleation hypothesis, also known as nucleation dependent polymerization hypothesis/
nucleated polymerization (SN). According to the TDR hypothesis, a correctly folded PrP^{C} can be unfolded and refolded into an altered conformation when it encounters a PrP^{Sc}, which acts as the template. To overcome the energy barrier of this conversion, the aid of a molecular chaperone denoted 'protein X' has been suggested [Telling et al. 1995]. The SN hypothesis states that there is a reversible monomeric equilibrium between PrP^{C} and PrP^{Sc} and several PrP^{Sc} need to form a stable nucleus/seed.
Once this threshold is reached, formation of amyloid (plaques/
deposits) and the shedding off of more infectious parties (seeds) takes place.

Both these hypotheses consider misfolded PrP^{C}, PrP^{Sc}, to be the infectious as well as toxic compound. Research by Sandberg et al. 2011 however 'tests' this by distinguishing two phases in the disease process; i.e. an uncoupling of infectivity and toxicity. How these findings could be explained within the TDR and SN hypotheses will be discussed. Different publications are combined to explain the models up for discussion. As especially neurodegeneration/toxicity is not well understood, looking at earlier models in light of this uncoupling might provide with new ideas on the disease process.

Template Directed Refolding hypothesis

* The heterodimer model

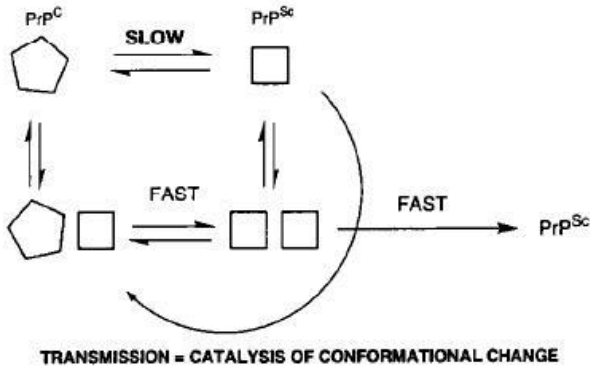


*Figure 7: Template Directed Refolding, heterodimer model. * Figure copied from Jarrett and Lansbury 1993.*

The heterodimer model is based on the idea of a conformational change of the healthy form (PrP-sen) into the pathogenic form (PrP-res), but does not require aggregate formation. PrP-res and PrP-sen form a eterodimers which dissociates to result into two PrP-res monomers [Cohen et al. 1994] (Fig. 7). PrP-sen can spontaneously convert to PrP-res and once PrP-res is present, its production is faster by interaction of PrP-sen and PrP-res. If the rate of degradation of PrP-res exceeds the conversion rate, PrP will be mostly in the protease sensitive state and any inoculation of PrP-res decays [Eigen 1996]. Whereas in the state where the rate constants have opposite relative magnitudes, there always is exponential growth of PrP-res. Nowak et al. 1999 state that "in this event, it becomes virtually impossible to juggle the rate parameters to produce a knife-edge where most of us seem to be in the healthy PrP-sen state, with only a few unlucky individuals victimized by PrP-res." This was already recognized by Eigen in 1996 which concluded that a non-cooperative autocatalytic fashion of prion formation is very unlikely as only an implausible parameter range is possible. Eigen in 1996 concluded that the co-operative autocatalysis model as well as the seeded nucleation model do not have this difficulty (this model will be explained further on).

* Co-operative autocatalysis

Apart from the heterodimer mechanism which postulates heterodimerization, it is possible that multimers are formed. This is a feature of the co-operative autocatalysis mechanism [Eigen 1996; Laurent 1997]. PrP^{C} and PrP^{Sc} together exist as mixed multimers and conversion of PrP^{C} to PrP^{Sc} takes place via allosteric interactions (Fig. 8).

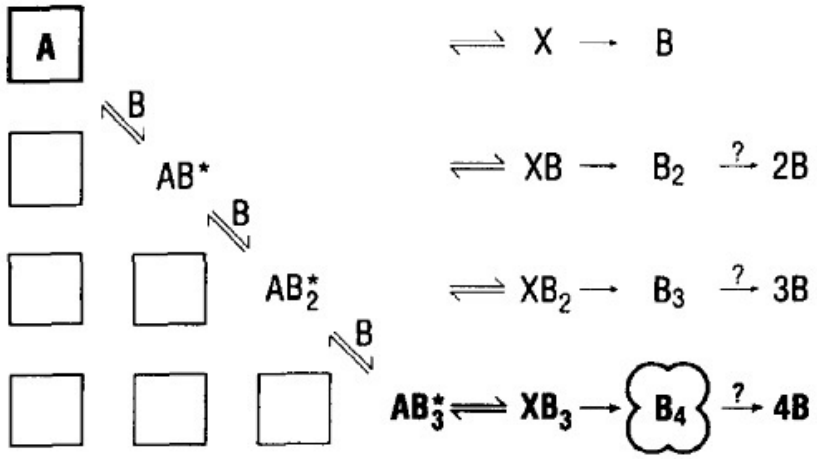


*Figure 8: A model for co-operative autocatalysis (as alteration of Prusiner's heterodimer model) * Figure and legend copied from Eigen 1996.*

In the co-operative autocatalysis mechanism, there is a thermodynamic equilibrium where also a B state (PrP^{Sc}) is favorable (which is different from the mechanism described above); it is assumed that the non-catalytic production of B is slow so its concentration cannot rise to a 'significant' level in the absence of infection [Eigen 1996]. Also, the assumption is made that linear catalysis is slow, so it does not successfully compete with metabolic removal of B. Eigen 1996 states that only higher order catalysis will compete with removal of B, provided that the level of [B] is high enough. The author state that co-operativity can introduce a threshold for [B]. When this is reached, the system switches from one steady-state condition to the other which is best illustrated in the case where only in the last step (between the trimer and tetramer in fig 8) the catalytic term becomes effective [Eigen 1996]. However, if the threshold is passed by infection, the system switches 'immediately' to the state where B prevails.

*** On-pathway intermediate**

However, an alternative to the heterodimer- and co-operative autocatalytic model is possible in which PrP^{C}, under stochastic fluctuations in the structure, can become a partially unfolded monomer (PrP*) which is an intermediate for the formation of PrP^{Sc} [Cohen et al. 1994]. PrP* can change back into PrP^{C}, be degraded or converted to PrP^{Sc}. Under normal conditions, the concentration of PrP* is low which means hardly any PrP^{Sc} is present. The authors state that the role of oligomerization remains uncertain. When infection takes place, PrP^{Sc}, the infectious particles, act as a template which promotes the conversion of PrP* to PrP^{Sc}. As PrP^{Sc} is insoluble, this process is not reversible which drives formation of PrP* and subsequently PrP^{Sc}. In the case of inherited prion diseases, a mutation in the PrP gene destabilizes PrP^{C} which predisposes to a conformational change into PrP* and thus increases the chances of PrP^{Sc} formation. PrP^{Sc} is then partially proteolysed (limited proteo-

lysis) to form a 27-30 kD fragment which can form amyloid [Caughey et al. 1991; Gasset et al. 1993; Safar et al. 1993]. It is suggested that in the case of sporadic prion disease, very rarely enough PrP* accumulates to produce PrP^{Sc}. This seems to be the case as transgenic mice overexpressing the PrP gene do develop prion disease (more often than wild type) [Westaway et al. 1994].

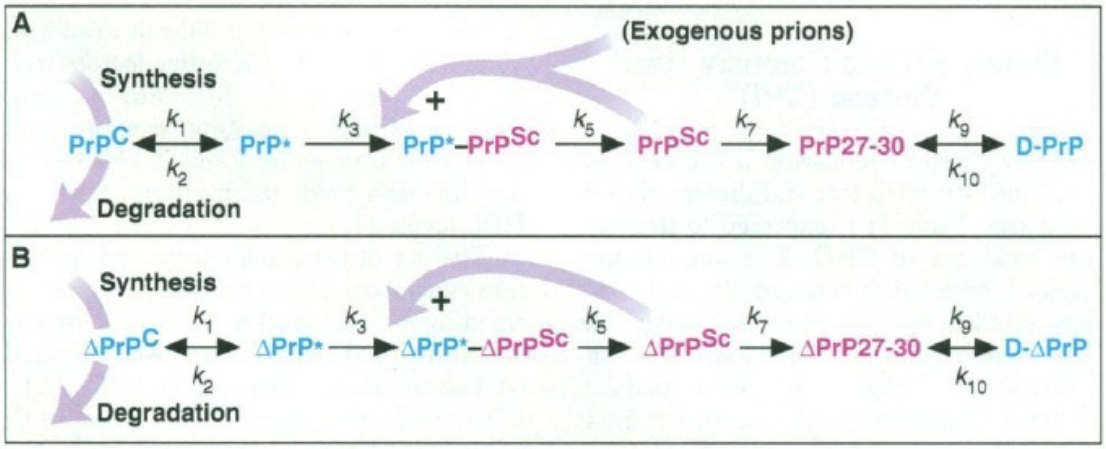


Figure 9: The conformational model for prion replication in the infectious and sporadic disease (A) and the inherited disease (B). In red, the infectious forms of PrP are shown whereas the blue PrP are non-infectious. PrP^{C} can reversible convert into a partially unfolded monomer PrP and upon encounter with PrP^{Sc}, can convert into PrP^{Sc}. After limited proteolysis, an N-truncated fragment remains denoted PrP^{27-30} which is said to be able to form amyloid. * Figure copied from Cohen et al. 1994.*

In the TDR model, the species barrier as well as the notion that there are different strains of prions can be explained according to the conformational selection model [Collinge 1999]: a certain degree of overlap between the infectious particle and PrP^{C} of the host determines if the host gets infected and if so, what the properties and disease course are. However, the existence of many different strains is, in my view, hard to explain in light of TDR where monomeric PrP^{Sc} is infective as well as toxic. As the infective compound is considered to be monomeric in the first two variants of TDR, there is no extra level of conformation to alternate which can be envisaged in SN. However, in the 'on-pathway intermediate' variant, amyloid formation is included but its role in toxicity is not explained [Cohen et al. 1994].

In all TDR models, PrP^{Sc} is considered to be the infective as well as toxic compound, no distinction is made and a role for protein aggregates is not included. Monomeric PrP^{Sc} is though to be able to serve as a catalytic surface for conversion of PrP^{C}. However, monomeric forms of PrP do not posses converting activity, PrPres polymers do as shown in cell-free and animal experiments [Caughey et al. 1995, 1997] as well as for yeast prions [Serio et al. 2000]. A threshold concentration of PrP^{Sc} was required for conversion which

does not correspond to a TDR mechanism [Caughey et al. 1995]. Also, the PrPres that is newly formed stays associated with PrPres aggregates and is not released as a soluble entity [Bessen et al. 1997]. Moreover, Prusiner et al. in 1981 showed that disruption of PrP^{Sc} aggregates greatly reduced infectivity. Is seems thus unlikely that monomeric PrP^{Sc} can be the infectious agent.

A partially unfolded monomer, denoted PrP*, has been introduced but has this ever been found? Serio et al. in 2000 elaborate on yeast prions and different mechanisms for conversion. The heterodimer model (in their publication denoted Monomer-Directed Conversion which does include aggregate formation (assembly)) is deemed unlikely for reasons mentioned above. Also, the authors have looked into the existence of a structural intermediate (called 'A' which is monomeric), which was not found. Either there is no such intermediate, or they conclude, the A structure is too rare to detect in solution during the time courses studied. These findings question the likelihood of a monomeric intermediate, as suggested in the 'on-pathway intermediate' model.

I believe that TDR as proposed by the hypotheses discussed here does not explain experimental findings. Other mechanisms have been proposed to accommodate new insights and findings.

The Seeded Nucleation hypothesis

* Griffith 1967

Griffith in 1967 proposed that protein subunits α can undergo reactions such as $\alpha_2 + \alpha \rightarrow \alpha_3$. Monomers thus assemble into multimers. It is assumed that $\alpha + \alpha \rightarrow \alpha_2$ cannot directly take place and α can thus only convert into a multimer structure when α_2 or larger multimers are present. It is proposed there are different monomers: the reactable subunit α and the stable subunit α'. It is suggested that the conversion from α' to α does not happen due to a too large free energy change and that all subunits are present as α' (Fig. 10).

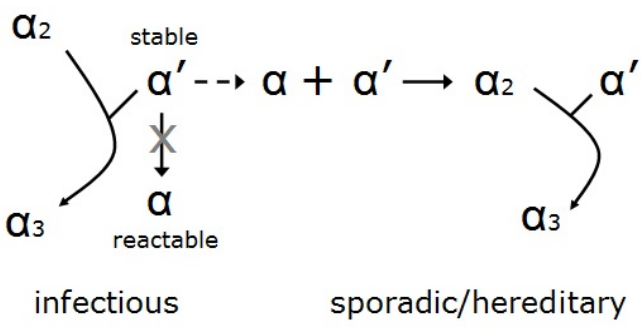


Figure 10: The protein, denoted α can be present in the stable (α) and reactable (α') conformation. Conversion to the reactable form usually does not take place. When infection takes place, a multimer is introduced which associates with a stable protein upon which conversion takes place. Sporadic/hereditary disease occurs when this first conversion does take place.

The method of propagation in this scenario is that of α' joining an α_2 template (which in this case is the infective agent) where it gets converted from α' to α and becomes part of the multimer, now α_3. Prion diseases can occur spontaneously when α' converts to α which subsequently heterodimerizes with an α' to convert this protein into α (to form together α_2), however this first step is a very rare event. Mutations that change the structure of the protein in such a way that conversion to other conformations is more likely than in wild-types can explain the hereditary/familial component. Infection occurs when multimers are ingested and thus the rare step of conversion of α' to α is not necessary.

*** SN as proposed by Jarrett and Lansbury, 1993**

The SN hypothesis was proposed by Jarrett and Lansbury in 1993 for Alzheimer's disease as well as for scrapie and has been expanded by others to include an intermediate form (Fig. 11). Psol (soluble, PrP^C) and P^U (transient unfolded state) monomers are in a rapid (according to Liemann and Glockshuber, 1998) reversible dynamic equilibrium with the concentration of P^U being very low in healthy cells [Tuite and Koloteva-Levin, 2004]. The SN model proposes that P^U (or the stabilized, amyloid, form denoted [P]) are not the nucleus for seeding polymerization, but that oligomeric form(s) of [P] are responsible for aggregation/amyloid formation [Tuite and Koloteva-Levin, 2004] which is referred to as primary nucleation. This formation of a PrP^{Sc} oligomer of critical size is the rate limiting step [Liemann and Glockshuber, 1998]. PrP^{Sc} precursors ([P]) are then further incorporated into the oligomer [Liemann and Glockshuber, 1998]. Secondary nucleations take place when a seed/nucleus sheds off and infects another cell [Orgel 1996].

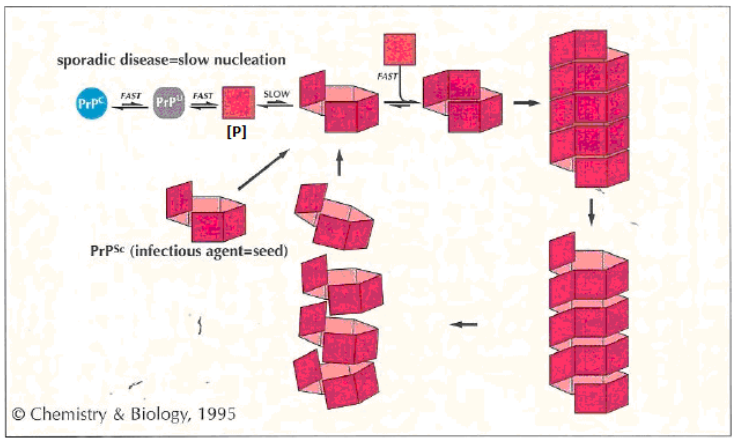


*Figure 11: Seeded Nucleation according to Lansbury and Caughey, 1995. Nucleus formation is the kinetic barrier to sporadic TSE, but bypassed by infection. Nucleus formation is very slow at concentrations slightly exceeding the critical concentration. Small increases in PrP concentration would greatly increase the rate of nucleation. Mutations in PrP that cause CJD may affect the unfolding equilibrium or association equilibria. * Figure and legend copied from Lansbury and Caughey, 1995.*

Three characteristics describe the SN hypothesis proposed by Jarrett and Lansbury in 1993:

Lag time: The formation of this nucleus can take a long time (the presence of a lag-phase). Studies of nucleation of a protiein involved in sickle cell anemia showed that the length of this phase is dependent on protein concentration [Hofrichter et al. 1974], at high concentrations, this lag-phase is not observed.

Critical concentration: When the system is in equilibrium, after polymerization is complete, monomers and high polymers predominate where concentration of the monomer is referred to as the critical concentration below which polymerization does not occur. The monomeric precursor PrP^{Sc} is under physiological conditions sparsely populated but if, at some point, this concentration exceeds a critical concentration (due to a shift in equilibrium), nucleation occurs, which is followed by a rapid propagation of PrP^{Sc} [Liemann and Glockshuber, 1998].

Seeding: Nucleation is the rate determining step at lower protein concentrations (lower saturation levels), addition of a seed/ preformed nucleus however leads to immediate polymerization (infection).

Jarrett and Lansbury showed in 1992 using a bacterial protein, that amyloid formation is seeded by preformed amyloid fibrils. Fibrils prepared from related sequence peptide analogs did not act as a seed. This suggested that complementarity between the seed and the amyloid peptide is required for growth [Come et al. 1993] which could be the reason for a transmission barrier between species. Liemann and Glockshuber in 1998 suggested that mutations in the PrP gene associated with inherited prion diseases may affect the conformational equilibrium between PrP^{C} and the monomeric PrP^{Sc} precursor (PU) and/or speed up the kinetics of the nucleation process due to slightly different conformation of PrP^{C}.

Thus in the SN hypothesis, the infectious units are not monomeric forms of PrP^{Sc} as these are supposed to be ubiquitous but the infective agent would be an ordered aggregate of PrP^{Sc} molecules [Aguzzi and Polymenidou, review 2004]. These are thought to have two active sites at either end of the linear chain [Rubenstein et al. 2007]. In order to explain exponential conversion rates, in this model it must be assumed that aggregates are continuously breaking to result in increased surface for growth [Weissmann 1999]. Conversion is suggested to be synonymous with integration of PrPsen into the aggregate [Nowak et al. 1999]. The model as propose by Jarrett and Lansbury in 1993 did not include an unfolded

intermediate. Perhaps the intermediate that was later added to the mechanism is necessary in order to explain sporadic/hereditary cases of prion disease whereas the introduction of a preformed seed does not require intermediates.

Herczenik and Gebbink, in 2008 explain propagation by SN and state that protein aggregation can result in different structures with intermediates (oligomers) which can be unordered amorphous aggregates or highly ordered fibrils which are called amyloid [Herczenik and Gebbink, 2008]. Thus aggregates can differ in their conformation and likely also in their properties and effects on the cell.

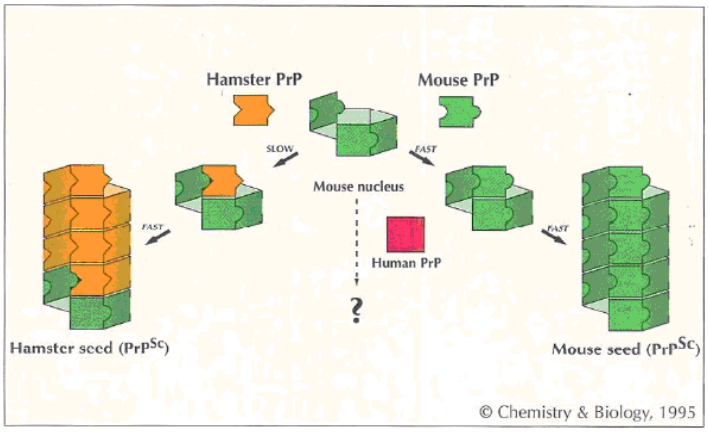
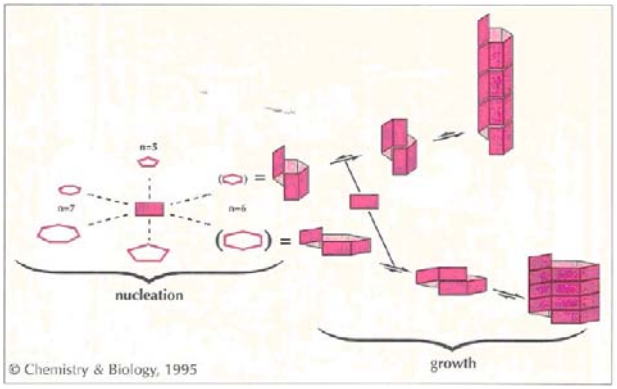


*Figure 12: A schematic representation of the species barrier which assumes that the conformation of the seed and the PrP^c of the host must be compatible. Intra-species seeding is most efficient and interspecies heterologous seeding can occur if the structural differences are small, whereas when the difference is too large, interspecies seeding may not be effective. * Figure copied from Lansbury and Caughey, 1995.*

Figure 13: Schematic representation of strains in SN hypothesis. Nuclei might be packed differently and size of seeds might also vary. The shape of the nucleus determines the shape of subsequent aggregates.
** Figure copied from Lansbury and Caughey, 1995.*



The fact that a species barrier exists can be explained by the same argument as in TDR (Fig. 12).
The phenomenon of prion strains is explained in the SN hypothesis: strains of PrP^{Sc} might differ in their packing of ordered aggregates (Fig. 13). These might depend on nucleus size and/or configuration and the conformation of monomeric PrP might be the same or also differ [Lanbury and Caughey 1995].

The SN hypothesis seems more likely in light of the finding that fragmentation of amyloid fibrils by sonication increased efficiency of seeding [Come et al. 1993; Jarrett and Lansbury, 1993]. Sonication fragments aggregates into smaller seeds which generates more growing-ends and increases the conversion into aggregates. This would not be seen if the TDR hypothesis would be correct as monomeric PrP^{Sc} is thought to be infectious, this does not require sonication to increase sites of conversion. This is supported by cell-free conversion experiments by Caughey et al. 1995 who found that the entity responsible for converting activity was many times larger than a soluble monomer and this required a threshold concentration of PrP^{Sc}. However, like TDR, the mechanism of neurodegeneration is not explained.

Toxic Intermediate/Toxic Side Product Hypothesis

Collinge and Clarke, in 2007, proposed a mechanism which explains experimental observations of uncoupling of PrP^{Sc} titers and toxicity mentioned earlier in this thesis. Two variations are described: the toxic templated intermediate (TTI) and the toxic templated side product (TTSP) model (Fig. 14). The main feature of these models is the uncoupling of PrP^{Sc} levels and pathology (and uncoupling of the propagating infectious agent and toxic species). PrP^{Sc} is assumed to be relatively inert, toxicity is established by PrP^{L} which is generated as an intermediate (in TTI) or a side product (TTSP) of PrP^{Sc}. It is proposed that when PrP^{C} is converted to PrP^{Sc} (which the authors deem to happen through template-assisted progression) the intermediate PrP^{L} is formed:

$$\mathrm{PrP^{Sc}} + \mathrm{PrP^{C}} \rightarrow \mathrm{PrP^{Sc}{:}PrP^{C}} \xrightarrow{k_1} \mathrm{PrP^{Sc}{:}PrP^{L}} \xrightarrow{k_2} \mathrm{PrP^{Sc}{:}PrP^{Sc}} \rightarrow \mathrm{PrP^{Sc}} + \mathrm{PrP^{Sc}}$$

*Equation 1: Mechanism of conversion of PrP^{C} to PrP^{Sc} involving an intermediate termed PrP^{L}. * Collinge and Clarke 2007*

There is no monomeric equilibrium between the cellular state PrP^{C} and another state (PrP^{Sc} or PrP^{L}) which is suggested in the SN hypothesis. Conversion is though to take place after hetero-dimerization where dimers exist as PrP^{Sc}: PrP^{C}, PrP^{Sc}: PrP^{L} and PrP^{Sc}: PrP^{Sc} consistent with TDR.

In cases where a subclinical infection is found, k1 (initial conversion) is slow, thus the level of PrP^{L} is low as k2 (maturation) is higher and processes PrP^{L} to PrP^{Sc} which will be abundant (thus high PrP^{Sc} levels are found without toxicity).
In cases of short incubation periods (such as in RML infection of tg20 mice, which over-express PrP^{C}), k1 is increased which results in a high level of PrP^{L}, k2 is in this case lower and thus PrP^{L} is not all converted to PrP^{Sc}. Thus low levels of PrP^{Sc} are found to coincide

with toxicity and lethality. If an infection is lethal depends on the ratio between k1 and k2 and the resultant presence of PrP^{L}. The introduction of a compound that is lethal and is different from the infective compound can explain how high prion titres are found to coincide with no/very late toxicity and vice versa. The authors however do not explain how k1 and k2 are established and on which factors these depend.

Also, the equation (Eq. 1) that the authors use to explain propagation seems to accept TDR as a mechanism of propagation. This is very unlikely as mentioned in the section about this mechanism. Within the SN hypothesis as explained so far, an intermediate that differs from the infective agent is hard to envisage. As a seed is made up of monomers of the same conformation (PrP^{Sc}), how is it possible that upon addition of a next monomer, this one does not convert to the same PrP^{Sc} but to another, here denoted PrP^{L}? As *in situ* studies of PrPres formation in prion infected brain slices showed that added monomers remain associated with the aggregate [Bessen et al. 1997], it seems very unlikely that this exact mechanism will be the case.

A proposed variation to this TTI is when PrP^{L} is not an intermediate in the synthesis from PrP^{C} to PrP^{Sc}, but a side product. When PrP^{C} is converted to PrP^{Sc}, this structure acts as a catalytic surface for PrP^{C} to PrP^{L} which is a toxic side product. In this mechanism, high PrP^{Sc} can be found to coincide with low toxicity when the conversion rate of PrP^{C} to PrP^{L} is slow. The authors however do not explain how it is possible that PrP^{Sc} can be a catalytic surface for conversion of PrP^{C} to PrP^{Sc} as well as to PrP^{L} and what determines if the former or latter is formed.

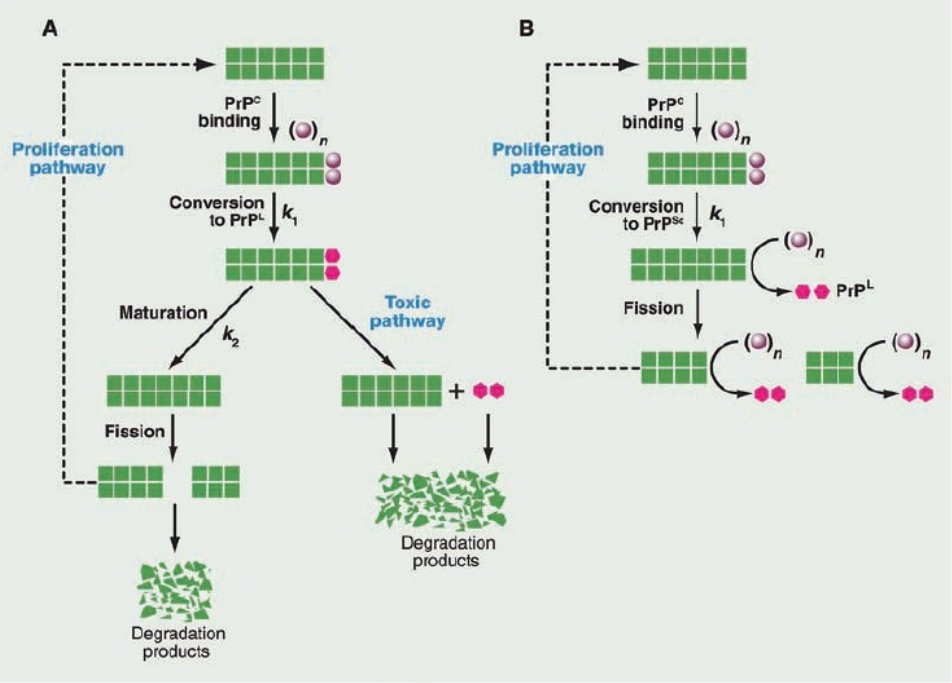


*Figure 14: A: Toxic Templated Intermediate model and B: Toxic Templated Side Product model as proposed by Collinge and Clarke, 2007. * Figure copied from Collinge and Clarke, 2007.*

EVALUATION OF SANDBERG ET AL. 2011

Now that the most important mechanisms of prion propagation as well as the results of Sandberg et al. 2011 have been discussed, I would like to speculate on the plausibility of the mechanisms and how uncoupling of infectivity and toxicity can be incorporated.

As mentioned, the TDR model is not supported by experimental findings. In this model, monomeric/multimeric PrP^{Sc} are thought to be infectious and the role of aggregates is not explained. The SN model seems more plausible and the existence of an intermediate/side product as proposed by Collinge and Clarke in 2007 could fit into this framework. The infective compound in this case would be a nucleus/seed of a certain size which incorporates monomers that undergo a conformational change. In phase 1 of Sandberg et al. 2011, the number of infectious units (PrPinf) increased in all mice until a plateau of a similar number of infectious units was reached. It is unclear from the publication whether these infectious units are all of the same size and if the size of infectious units changes during phase 1 and phase 2. This should have been assessed. It is conceivable that even though the number of infectious units was similar in all mice, the size was not. The increase in number of infectious units was not dependent on $[PrP^{C}]$ but probably on the number of infectious units in the inoculum since

studies in Syrian hamsters revealed that there was an inverse correlation between the length of the incubation time and the logarithm of the dose of inoculated prions [Prusiner et al. 1982b; Prusiner et al. 1980]. However, how and when the proposed pathway shift is made from phase 1 to phase 2, is not explained in the 'PrP^{L}' model posed in 2007 neither by Sandberg et al. 2011 (the same group) in their recent study. Sandberg et al. 2011 suggested that production of PrP^{L} starts at the moment infectivity reaches a plateau (Fig. 6). I deduct from their explanation that from this point on, no infectious units are produced (as the number of infectious units did not further increase) and production of PrP^{L} commences. There is thus a switch from PrPinf being a catalytic surface for PrPinf (PrP^{Sc}) production, to PrPinf being a catalytic surface for PrP^{L} production. The authors suggest PrP^{L} to be oligomeric forms of PrP^{Sc}, but do not explain the mechanism involved nor what determines the moment of switching.
It is unlikely that production of toxic species does not start before the infectivity has reached a plateau. Weissmann in 2004 discusses findings that the number of PrP molecules in scrapie infected brain homogenate is several orders of magnitude larger than the number of infectious units. The author speculates that perhaps the infectious process is very inefficient, the infectious unit a large aggregate or a minority component of the homogenate and other isoforms make up for the rest. Indeed the infectious units are aggregates: Silveira et al. 2005 showed that the most efficient initiators of TSE disease in Syrian golden hamsters were particles consisting of 14-28 PrP molecules. These are most infective and show highest converting activity whereas these activities in large fibrils were much lower and nearly absent in oligomers ≤ 5 PrP molecules. It is likely that the isolates do not consist solely of one 'sort' of misfolded protein. Some units might be infectious whereas others are not (yet).

It is conceivable that indeed there are different aggregates of PrP^{Sc} of which some might act as infectious units whereas others do not, but instead the latter have a detrimental effect on the cell. The latter could be smaller oligomers which perhaps are produced by cleavage of larger aggregates as a by/post-product of PrPinf formation, and these oligomeric species are toxic as discussed by Novitskaya et al. 2006. For some reason, these oligomeric species do not possess converting properties (and are not picked up in the assay that determines infectivity which means these might be PK sensitive). It is conceivable that a conformational change follows shedding off of these oligomers from their parent aggregate since they now are not in a fibrillar aggregate. This gives them freedom to be present in another, slightly different, non-fibrillar conformation (perhaps by interaction with another protein such as a chaperone). This conformation possesses properties different from

those of fibrillar aggregates and this result in toxicity (Fig. 15A). However, the oligomer, in this case, is impaired in its ability to grow by addition of monomers (as discussed in Nguyen et al. 2007, who in case of Alzheimer's state that smaller oligomeric non-fibrillar species are on-pathway to amyloid formation). PrP^L in case of the SN as discussed here, is a by/post product. However, as proposed by Nguyen et al. 2007, it is conceivable that PrP^L indeed are non-fibrillar oligomers which are on-pathway to amyloid formation, a by/post-product, or maybe even both. The SN as explained so far then needs to be revised to include this intermediate/extra step which then resembles the model proposed by Collinge and Clarke, 2007. The question is whether these smaller oligomers are stable as the SN hypothesis states that a nucleus of a critical size needs to be formed which is stable, whereas smaller nuclei fall apart [Nowak et al. 1999]. However, smaller oligomers have shown to be toxic so these seem to be stable (at least for some time) [Novistkaya et al. 2006]. Novitskaya et al. 2006 suggest that in this case, aggregate forming should protect from the toxic effects of oligomers as these aggregates sequester oligomers. Also, it is conceivable that the non-fibrillar oligomers might be on-pathway to forming larger non-fibrillar aggregates which are toxic after having crossed a size-threshold (Fig. 15B). The formation of fibrillar aggregates would be a storage mechanism (as mentioned above) and the non-fibrillar aggregates result in cell demise, as shown for Aβ in Alzheimer's Disease [Lin et al. 2001; Gibson et al. 2004]. However, the demonstration of Novitskaya et al. in 2006 that mature amyloid fibrils also proved toxic to cultured cells shows that the matter is far more complex. The authors state that it is likely that conformational variation in fibrils determines properties of neurodegeneration and different amyloid structures exist [Alvarez-Martinez et al. 2011].

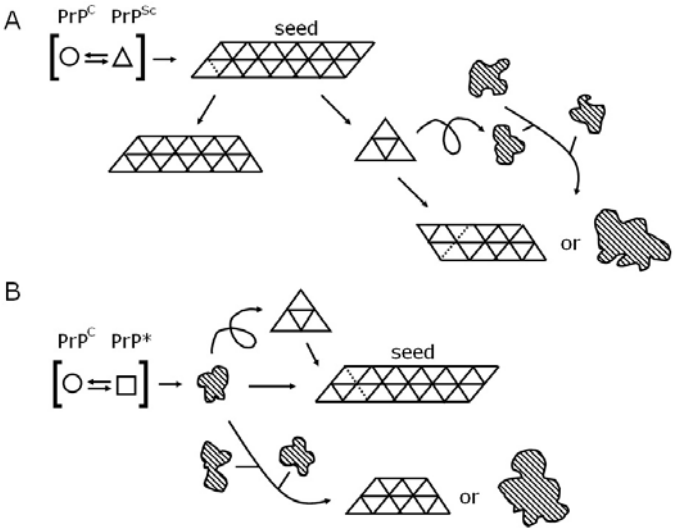


Figure 15: A: The cellular form and the scrapie form (or an intermediate, not shown) are in equilibrium. Upon infection, the seed (fibrillar oligomer) grows by addition of monomers. Upon breakage, when an oligomer of a certain size(s) dissociates, conversion might take place to non-fibrillar oligomeric specie which might be toxic (shaded). These oligomers might form larger non-fibrillar oligomers (shaded) which are toxic, or are captured into larger fibrillar oligomers which are not toxic but are able to grow. B: Non-fibrillar oligomers are formed from a non-infectious form of PrP, PrP, which can spontaneously convert into fibrillar oligomers with subsequent association by a seed/fibrillar oligomer. The non-*

fibrillar oligomers can perhaps also form fibrillar aggregates which can grow and are non-toxic and/or form non-fibrillar, toxic oligomers.
The fact that fibrils can not be approached as one entity that can either be 'long' or 'short' is supported by findings of Lee et al. 2011 who showed two different structures of PrP within ordered aggregates. These differ in their secondary, tertiary and quaternary structure. A decrease in size of the amyloid fibril could either enhance or abolish their toxic effects on Chinese Hamster Ovary and human melanoma cell line, this depended on the structure. This adds another dimension to the problem: apart from non-fibrillar and fibrillar aggregates, the fibrillar type shows (at least) two variants with opposite behavior. However, these two different structures have been generated *in-vitro* which does not necessarily mean their existence *in vivio*.

It is proposed that the toxic effects of non-fibrillar aggregates are mediated by their ability permeabilise the plasma membrane to ions, shown for prions as well as Aβ and IAPP [Kayed et al. 2004] or by forming a membrane pore which results in membrane impairment as shown by exposing a synthetic membrane to hamster prions [Paulis et al. 2011].

Unfortunately, it is not known if the mice in the recent study presented with aggregates in their brain and what patterns PrP deposits might show as no microscopic studies have been performed. Because of this, I share critique by Wickner 2011 (in the same issue as Sandberg et al.): indeed infectivity was measured at several time points whereas only one value for the toxic species was measured; namely when clinical disease started. The authors assumed that the same amount of toxic species was lethal to all types of mice used in the study which might not be the case. Wicker speculates that rapid accumulation of a smaller amount of the toxic species might kill the transgenic PrP^{C} over-expressing mice whereas wild type/hemizygous mice that more slowly accumulate toxic species somehow might, to a degree, adapt and subsequently die when exposed to a greater amount of toxic species. This might be attributed to amyloid formation. This process has shown to take a considerable amount of time [personal communication with Prof. Dr. Rozemuller] and it is conceivable that PrP^{C} over-expressing mice produce the toxic species at a faster rate (which can be deduced from the shorter plateau phase) than wild types and are somehow not able to sequester precursors in 'harmless' fibrillar amyloid.

I assume that as the identity of the toxic species is not fully understood, it was not possible to also measure the presence and growth in number of this. However, fixing the brains of the mice and staining for PrP^{C}, PrP^{Sc} and dyes specific for amyloid and oligomeric species, followed by microscopic inspection would have

provided a lot more information on the disease course. In vivo multiphoton microscopy imaging of plaques (or other structures) could prove to be very informative in formation and dynamics of these structures as it has in an Alzheimer's disease model (Christie et al. 2001, mouse experiments).

Also other cellular factors probably play a part in the disease process such as rate of degradation/clearance of misfolded protein which could become overloaded much sooner in PrP^C over-expressing mice. This idea is illustrated by what Nowak et al. in 1999 proposed, namely that the sudden onset of disease might be a consequence of a redundant organization of brain function and only when a threshold is crossed, this ability to compensate is lost and clinical disease starts and progresses quickly. PrP^C over-expressing mice might not have the time to compensate and therefore, die early whereas mice expressing normal levels of PrP^C also build up toxic species, but as this process is slower, manage to cope for some time which would depend on PrP^C and the threshold for disease onset may be at higher concentrations of toxic species.

Wickner in his commentary in Nature, 2011, suggests that amyloid might clog up some intracellular compartment after internalization, such as endosomes which results in cell death. As the process of conversion of PrP might take place much faster in PrP^C over-expressing mice, these compartments soon are overloaded whereas WT and $Prnp^{+/-}$ mice might be able to (partially) clear these structures. The author also suggests that toxicity might be proportional to total length of filaments. Chaperones are according to the author likely to be constantly active to cleave prion filaments. In hemizygous mice, the few filaments would be cleaved into many pieces which are infective whereas PrP^C over-expressing mice may have larger filaments of greater toxicity but lower infectivity per mass. The infectivity of all mice was similar at the plateau which means that hemizygous mice might have many small fragments whereas over-expressing mice have many large fragments. The observed pathway switch can mean the passing of a length-threshold for filaments which changes the property of the aggregate from infectious to toxic. The question then arises as to where exactly the cut-off is for infective vs. toxic length. And, is length the only determinant or perhaps smaller fragments are present in a different conformation which show different properties.

However, this is relationship of toxicity which is proportional to filament length is conflicting with the findings of Novistkaya et al. in 2006 that non-fibrillar oligomeric species (which are on-pathway or a by-product of amyloid) seem to be toxic and it was proposed that aggregation sequesters these and thus counteracts toxicity. As

aggregates are not seen in all disease cases (cases with a short disease period) [personal communication with Prof. Dr. Rozemuller], it is more likely that non-fibrillar oligomers indeed are toxic. This is in accordance to what Hill and Collinge proposed in 2003: a soluble or oligomeric conformer of PrP^C is the toxic compound. The authors make the comparison with Alzheimer's disease where the soluble pool of Aβ peptide (opposed to the insoluble pool associated with plaques in the brain) has been shown to be the principal determinant of the severity of neurodegeneration [McLean et al. 1999]. I believe that apart from the toxic oligomers, non-toxic oligomers are present which can form amyloid. Perhaps the aid of a certain protein or clearance mechanism is necessary in order to form more organized oligomers as some kind of rescue-mechanism to safely store misfolded proteins. In cases of PrP^C over-expressing mice, this protein is depleted which results in more formation of non-fibrillar oligomers and thus toxic levels are reached much earlier compared to WT. In order to study kinetics of the toxic compound, it first needs to be identified. Then, specific antibodies might shed light on cellular localization as well as dynamics of the amount/number of these toxic/non-toxic species. When different structures are identified and can be measured, graphs similar to figure 6 can be made, but now showing behavior of the different sub-groups: fibrillar, non-fibrillar, grouped by size, perhaps also by localization.

An alternative is that in PrP over-expressing mice, the toxic oligomers soon accumulate as the cell can not process these fast enough to store/degrade them. WT mice however have a much smaller load of these toxic oligomers and perhaps can process these indefinitely. However, the storage product accumulates and at a certain moment, induces cell death. This would mean that the induction of cell death is different in PrP^C over-expressing mice compared to WT and hemizygous mice. At the moment it seems that the toxic, and probably also the infectious, species are a heterogeneous group in respect to size and conformation. Analyses of many different forms of PrP would provide with much more information from which possible mechanisms might be deducted.

Claudiu Bandea, an opponent of the prion-hypothesis, has not refrained from criticizing the recent study. The author supports Wicker in his critique regarding measurement of toxicity as Bandea states that no pathological markers have been measured. I share the critique by Bandea that what Sandberg et al. state, namely that toxic species are produced from the moment phase 2 starts, is not supported by their data. Bandea however goes one step further and disputes the notion that Sandberg et al. conclude that infectivity is not produced during phase 2. However, I believe that Sandberg do

not clearly state that production of infective species ceases in phase 2, it is conceivable that either the number of infectious units does not increase (as shown by measurements) but that they still grow in size, or perhaps in phase 2, there is an equilibrium in infective species where the rate of degradation/conversion to toxic- or byproduct is similar to the rate of production. As it is not clear what size the infectious units are, I believe it is valuable to study this as well as turn-over rates of these units.

CONCLUDING REMARKS

It is clear that after decades of study, the functioning of prions is still enigmatic and we seem to have so far uncovered only parts of the involved processes. The uncoupling of infectivity and toxicity seems clear but the identity of especially the toxic compound and the process of neurodegeneration are not understood.

I believe most data points to seeded nucleation as a mechanism of propagation of infective units, however, if, how and when the pathway switch proposed by Sandberg et al. 2011 takes place, is not clear. I have made some suggestions to explain an uncoupling of infectivity and toxicity. The nature of the toxic species however is not established as well as the mechanisms leading to neurodegeneration. There are more other proteins and processes involved in the disease process and neurodegeneration than mentioned in this thesis such as the role of apoptosis, the signaling function of PrP^C, effects of copper as well as possible ER stress and mitochondrial demise and involvement of LRP1, a transmembrane receptor that is required for copper mediated endocytosis of PrP^C in neuronal cells [Taylor and Hooper, 2007]. It is conceivable that depletion of certain proteins occurs after prion infection which might result in either less clearance of infective/toxic species or somehow the mechanism of conversion is changed in a way that allows production of toxic species. Also, PrP is shown to interact with other proteins in the plasma membrane such as heparan sulphate proteoglycans [discussed by Guillerme-Bosselut et al. 2009] and interference with some of these interactions has shown anti-prion activity [Adjou et al. 2003]. The involvement of chaperones and proteasomal degradation are not clear but recent findings in human embryonic stem cells that show rapid up-take and clearance of prions, show that apparently these cells are able to deal with infection [Krejciova et al.2011]. Comparing these specific cells to adult neuronal cells might provide with new therapeutic targets. Yeast and fungi also possess prions and different chaperones are involved in propagation by breakage of prion polymers. Inhibition of this these chaperones, an thus breakage, resulted in loss of prions whereas over-expression of one of the chaperones also led to loss of prions but via another mechanism, and just for one of the several types of prions that the studied yeast can have [reviewed by

Kirkland et al. 2011]. Perhaps similar mechanisms are at work in mammals. It is thus of great interest to detect possible chaperones in mammals and determine their effects on prion propagation.
Studies that aim at measuring changes in gene/protein expression during infection and neurodegeneration, such as the study by Tamgüney et al. (2008), will provide with a more complete picture of the effect of infection on the cell. It is also conceivable that the mechanisms of cell death/toxicity in PrP^C over-expressing mice differ from that in wild-type/hemizygous mice.

While writing this thesis, I have accepted the protein-only hypothesis but until today there are people who challenge this and support hypotheses which involve a virus in one way or the other [Manuelidis, Bandea]. These hypotheses, according to proponents, are able to better explain many features of prion disease compared to the prion-hypothesis such as strain variation and inter-species transmission patterns. However, as conversion of the native protein into the pathogenic form is shown possible *in vitro* in a chemical process, theories including a virus are redundant.

As mentioned in the introduction, prion diseases are not the only maladies resulting from misfolded protein and subsequent aggregates. Diseases such as Alzheimer's, Parkinson's and type II diabetes share many similarities. Apart from studying disease out of sheer curiosity, new findings will prove extremely valuable in light of the fact that many people suffer from before mentioned conditions.

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