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Mechanisms of Cell Death in the Transmissible Spongiform Encephalopathies

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1. Introduction

The transmissible spongiform encephalopathies (TSEs) constitute a family of fatal, neurodegenerative diseases, including scrapie in sheep, chronic wasting disease (CWD) in deer and elk, bovine spongiform encephalopathy (BSE) and a range of human disorders, such as Creutzfeldt-Jakob disease (CJD), kuru and fatal familial insomnia. The archetypal TSE disease is scrapie of sheep and goats, which has been present in the UK flock for over 200 years as a result of both horizontal and vertical transmission. The most prevalent TSE disease of humans is sporadic Creutzfeldt-Jakob disease (spCJD), which affects 1-3 individuals per million worldwide. A new form of CJD, known as variant CJD (vCJD), was diagnosed in humans in the mid 1990s and it is likely that vCJD was contracted by consumption of contaminated beef, since this disease is indistinguishable from BSE on transmission to a panel of mice (Bruce et al., 1997). To date, there have been 175 cases of vCJD in the UK and a further 49 cases across 11 other countries (www.eurocjd.ed.ac.uk, data correct as of Aug 2011*).

During pathogenesis of TSE disease the principal molecular event is the conformational rearrangement of a normal, host protein called the prion protein. The normal form of the prion protein, PrP^C, misfolds to a form known as PrP^{Sc}. PrP^{Sc} is insoluble and partially resistant to digestion by proteolytic enzymes that would usually recycle incorrectly folded proteins. PrP^{Sc} therefore accumulates in proteinaceous aggregates, including plaques and fibrils. The prion protein is ubiquitously expressed, but is most abundant in the central nervous system (CNS). Hence accumulation of PrP^{Sc} occurs principally in the brain, but peripheral lymphoreticular tissues can also accumulate proteinaceous deposits. The prion hypothesis suggests that PrP^{Sc} is the infectious agent in TSE diseases and that it catalytically causes nascent PrP^C molecules also to misfold (Prusiner, 1998). TSEs exist as discrete strains of disease, which can be stably passaged in suitable hosts resulting in differences in incubation time, clinical signs and pathology. It is suggested that PrP^{Sc} exists in different conformations, which encode the information necessary to transmit each disease and cause the strain-specific pathology (Prusiner, 1998). As a result of the critical involvement of the prion protein in TSEs, these disorders are also known as prion diseases.

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At clinical end point of TSE disease, there is characteristic vacuolation in various areas of the brain, the exact locations of which depends on the infecting strain. Loss of neurons can also be detected at late stages of disease as can alterations in membrane morphology. Several excellent reviews cover neuropathology of animal (Jeffrey et al., 2011) and human (Kovacs & Budka, 2009) diseases that is evident on post mortem examination. In general, there are good correlations between disease, neurodegeneration and prion protein aggregation in many TSE diseases, which has led to suggestions that PrP^{Sc}-containing aggregates are directly toxic to neurons. *In vitro* studies largely support this conjecture, but the evidence *in vivo* is less convincing. Even assuming that a misfolded form of the prion protein is responsible for neurotoxicity, the mechanisms that initiate the cascade leading to neuronal loss are unknown. It is also unknown whether loss of function of PrP^{C}, as it is sequestered from the cell surface into proteinaceous aggregates, plays a role in rendering neurons susceptible to degeneration. Reactive astrocytosis is evident during the clinical phase and whilst time course studies have also suggested that astrocytes are activated at earlier stages, it is not known to what these cells are responding. In this chapter we review briefly the state of knowledge of the processes leading the neurodegeneration in TSE diseases, with a particular focus on the earliest detectable events.

2. Early morphological events in TSE-induced neuronal loss

In both prion diseases and other neurodegenerative disorders, the mechanisms leading to neurodegeneration remain particularly poorly understood. As mentioned above, the clinical phase of a variety of natural prion diseases has been studied, which has produced descriptions of the targeting of pathology, including the localisation of PrP^{Sc} deposits and of characteristic vacuolation and spongiform alterations. Substantial neuronal loss occurs by terminal endpoint, but it has become clear that loss of neurons is a relatively late development in the progression of pathology. In common with other neurodegenerative diseases, at later time points there are characteristic abnormalities in a range of normal neuronal molecular processes; this includes defects in ion homeostasis, aberrant mitochondrial morphologies and function, increased production of reactive oxygen species, endoplasmic reticulum stress and reduced proteasome function. Many of these homeostatic defects are thought to drive each other and it is therefore not clear which, if any, is the initiating factor. Thus, gross defects in several biochemical pathways represent the end stages of disease, but to determine causal mechanisms, it is necessary to describe in molecular and morphological detail the earliest stages of the neurodegenerative process. In naturally-contracted diseases, such descriptive studies are frequently not possible because (i) it is difficult to diagnose disease in advance of clinical signs (ii) outbred animals and humans can show significant variability in specific responses to disease and (iii) it is often impossible to know how and when individuals became infected. To remedy this situation, much use has been made of rodents as models of prion disease; C57BL/6 mice infected with the ME7 murine scrapie strain is the experimental system that has been studied in the greatest detail. There are clear advantages in using experimental prion diseases as a model, since the disease begins and ends at defined points (inoculation and death) and the homogeneity afforded by inbreeding produces standardised results. One caveat to murine models is that it appears that not all aspects of TSE disease in rodents are replicated in natural disease of large animals (Jeffrey et al., 2011). Nevertheless, some key findings from study of ME7 infection of mice are depicted graphically in Figure 1 and discussed below.
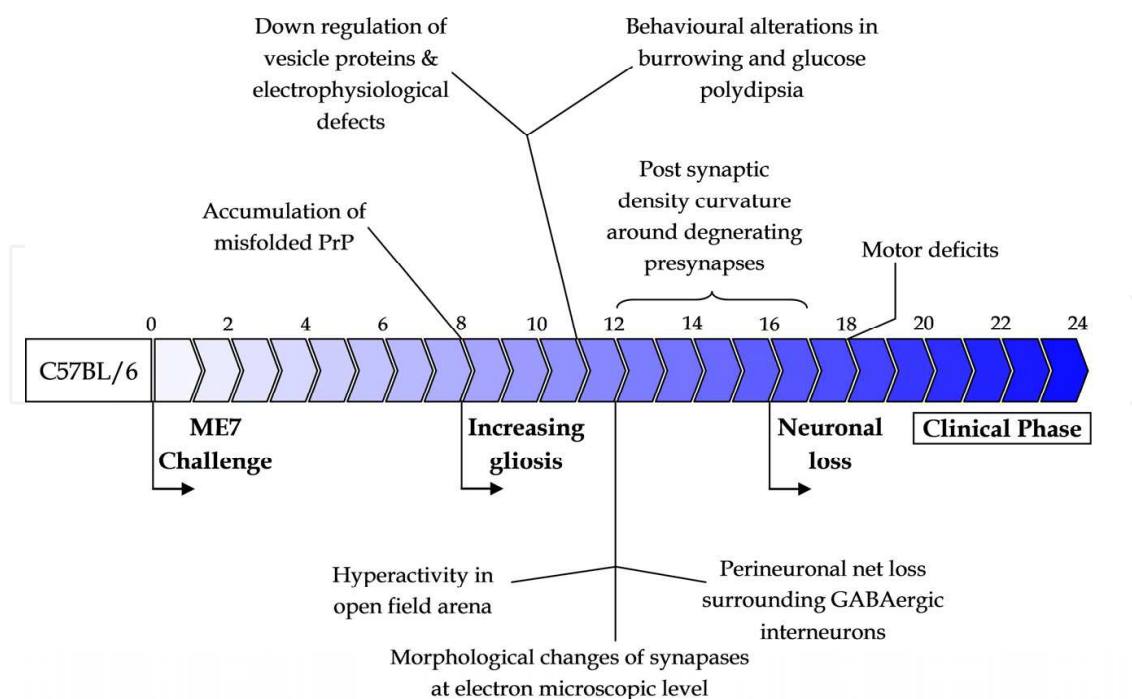
Fig. 1. Schematic timeline, in weeks, of pathological and behavioural events during infection of C57BL/6 mice with the mouse-passaged prion stain ME7. Information contained in this figure has been abstracted from multiple publications, referenced in section 2.

2.1 Synaptic degeneration precedes neuronal loss in prion disease

Recent studies of the pathogenesis of the ME7 strain of murine scrapie have allowed the identification of synaptic deficits within the hippocampus of C57BL/6 mice that occur well before neuronal loss can be observed (Betmouni et al., 1999, Cunningham et al., 2003, Guenther et al., 2001, Jeffrey et al., 2000). From roughly half way though the incubation period, synaptic deficits can be characterised at a molecular level by a loss of integral synaptic vesicle proteins and reduced synaptophysin staining (Cunningham, et al., 2003, Cunningham et al., 2005, Gray et al., 2009). Importantly, molecular changes appear to correlate with functional deficits, since electrophysiological abnormalities have also been observed within a similar timeframe (Chiti et al., 2006). At week 12, an accumulation of electron rich material within the pre-synapse in the CA1 region of the hippocampus was observed by use of electron microscopy, specifically between CA3 pre-synapses and CA1 post-synaptic densities within the Schaffer-Collateral pathway (Siskova et al., 2009). As the disease progressed, a distinct curvature of the post-synaptic densities around the degenerating pre-synapses could be visualised (Siskova, et al., 2009), potentially an attempt to maximise synaptic transmission. In addition, a loss of perineuronal nets surrounding GABAergic interneurons of the hippocampus coincided with a reduction in synaptic plasticity at early time-points (week 11/12) (Franklin et al., 2008). Early synaptic changes are a feature of other strains of murine disease, suggesting that these events may be early pathological markers of a TSE infection (Siso et al., 2002), at least in rodents. Synaptic dysfunction also appears to be a consistent, early pathological sign in many other neurodegenerative diseases, but there are suggestions that the exact morphological changes seen may differ depending on whether the insults to synapses are caused by processes

leading to intra- or extra-cellular protein deposits. In addition, synaptic dysfunction in prion diseases differs from that seen during Wallerian degeneration in the periphery, in which axons are dissected or otherwise compromised and presynapses retract (Gillingwater et al., 2003). In prion diseases, loss of synapses appears to be followed by a retraction of the dendritic spine, but whether loss of any given synapse impacts on neighbouring synapses and ultimately on the respective cell body remains to be determined.

The early loss of synapses in prion disease must occur in response to a disease-associated molecular event or biochemical pathway. It is possible that this event may be the beginnings of the misfolded protein cascade, since in C57BL/6 mice infected with ME7 scrapie the accumulation of PrP^{Sc} can be detected at week 8, before the first observable signs of synaptic defects. The first deposits of abnormal PrP accumulate in the dentate gyrus of the hippocampus, subsequently spreading to encompass the CA3 sub-region of the hippocampus (Gray, et al., 2009). This suggests a progression of PrP^{Sc} formation along the Mossy-Fibre pathway, connecting the dentate gyrus and CA3 field, and a subsequent pathological dysfunction of CA3 neurons leading to degeneration of CA3 pyramidal cell pre-synapses in the CA1 region along the Schaffer-Collateral pathway. In the majority of prion diseases that have been studied in detail, PrP^{Sc} accumulation is one of the earliest detectable pathological signs and precedes, or is concurrent with, cellular or synaptic changes. These results suggest a causative correlation between the initial signs of PrP conversion and synaptic dysfunction. This raises the question of whether neurodegenerative processes are also similar in other prion disease models, particularly those that have small quantities of misfolded PrP present at the clinical end point (Barron et al., 2007). In the majority of cases, time course studies of such disease models have not been performed in sufficient detail to dissect the earliest pathological events. It is clear, nevertheless, that synaptic dysfunction and degeneration occur well before neuronal loss is observed in TSEs.

2.2 The role of glia in prion disease-induced neurodegeneration

Although neuronal death in TSEs is the most widely recognised pathological manifestation at a cellular level, alterations in non-neuronal cells are also apparent occurring alongside the first obvious signs of PrP^{Sc} accumulation in the brain. Reactive astrogliosis, exemplified by up regulation of *Gfap*, can be seen in various areas of the brain (Betmouni et al., 1996, Cunningham, et al., 2003). An increased understanding of astrocytes suggests that these cells have an integral role in maintaining homeostatic functions within the CNS (Butt et al., 1994, Chang Ling & Stone, 1991, Ransom et al., 2003, Robinson & Dreher, 1989, Slezak & Pfrieger, 2003). Astrocytic processes come into close contact with synapses (Bushong et al., 2004, Grosche et al., 1999) forming a 'tripartite' between the pre and post synaptic elements and the fine astrocytic processes (Araque et al., 2009). Astrocytes can undergo excitatory mediated release of chemical neurotransmitters as a result of increases of intracellular Ca^{2+} concentrations in the astrocyte cytoplasm (Kreft et al., 2009). Reactive astrogliosis is thought to play a neuroprotective role during acute brain injuries, for example during cerebral ischemia (Pekny et al., 2008), but it is not clear whether the activation of astrocytes is also neuroprotective during chronic infections, such as TSE diseases.

Microglia also exhibit an activated morphology prior to and concurrent with neurodegeneration, however, this doesn't appear to represent the classic inflammation one may expect during infection with classical pathogens (Perry et al., 2002). Instead, a concept

of 'microglial priming' is thought to occur. Activated microglia produce an anti-inflammatory phenotype in response to ongoing TSE pathology, but subsequent systemic insults can elicit a rapid inflammatory response, initially by increases in IL-1β (Perry et al., 2007, Perry et al., 2003). Since the microglial response is not associated with classic inflammation, the role that these cells play in neurodegenerative disease remains unknown. It has been hypothesised that microglia have a neurotoxic role in neurodegeneration (Block et al., 2007) but other studies suggest microglia could be neuroprotective (Solito et al., 2010). There are also suggestions that microglia are not involved in the neurodegenerative process at all, but that degeneration is a neuron-autonomous process, at least at early stages (Perry & O'Connor, 2010). For both astrocytes and microglia, it remains unknown what these cells are responding to and whether this response aids or is detrimental to neuronal health. However, their activation at around the time that PrP^{Sc} deposition can first be observed suggests that they respond to the ongoing conversion process, to the accumulation of PrP^{Sc} itself or to the changes that PrP conversion and/or PrP^{Sc} deposition elicits in cellular mechanisms or synaptic morphology, plasticity and function. Mice in which PrP^{C} expression is restricted to astrocytes are susceptible to TSE infection (Raeber et al., 1997) suggesting that these cells are important in replication of PrP^{Sc} as well as in responding to its presence. In many studies using the ME7 murine scrapie strain, there exists strong correlations between the initial accumulation of PrP^{Sc} and neurodegeneration suggesting a key role for misfolded PrP in the mechanism of neuronal degeneration. This raises the key question of whether abnormal PrP isoforms are neurotoxic and, if so, what their molecular structures are.

3. Molecular mechanisms underlying degeneration of neurons

Prion protein deficient mice are resistant to TSE infection (Bueler et al., 1993, Manson et al., 1994b), demonstrating that PrP^{C} is required for disease. However, it is unclear what property of PrP is important for pathology: whether the PrP^{Sc} that accumulates during disease is actually toxic to neurons directly, whether the loss of PrP^{C} plays a role in rendering neurons susceptible to toxic insults (either involving PrP^{Sc} or not) or whether the ongoing process of agent replication compromises normal neuronal homeostasis. Although PrP^{Sc} accumulation appears to precede neuronal loss in ME7 scrapie, there are reasons to suggest that the accumulation of misfolded PrP is not responsible for neurotoxicity directly. For example (i) neurons which lack PrP^{C} do not degenerate in the presence of infected graft tissue rich in PrP^{Sc} (Brandner et al., 1996) and (ii) a variety of models exist in which levels of PrP^{Sc} and neuronal loss are poorly correlated (Barron, et al., 2007, Baumann et al., 2007, Chiesa et al., 1998, Flechsig et al., 2003, Hegde et al., 1998, Lasmezas et al., 1997, Li et al., 2007, Ma et al., 2002, Muramoto et al., 1997, Piccardo et al., 2007, Shmerling et al., 1998). But if classical PrP^{Sc} is not neurotoxic, then what is the toxic species?

3.1 Neurotoxicity of different aberrantly folded PrP isoforms

Considerable morphological heterogeneity can be observed in the protein deposited *in vivo* and recombinant PrP (recPrP) also exhibits conformational flexibility *in vitro*. There are also reports of aberrant cell biological behaviour of PrP^{C} at various stages of its cellular life-cycle, as depicted in figure 2, and these factors make pinpointing the neurotoxic entity rather challenging. Recent studies seem to support the idea that relatively small, (pre-fibrillar?) oligomeric protein species are highly neurotoxic (Bucciantini et al., 2002, Caughey &

Lansbury, 2003, Novitskaya et al., 2006, Simoneau et al., 2007, Zhang et al., 2010) and this appears true not just for prion diseases but also for other neurodegenerative protein misfolding diseases, further suggesting that common mechanisms of neurodegeneration may exist. RecPrP preparations have been used to investigate what the mechanisms underlying neuronal death may be, but *in vitro* studies such as these come with their own set of limitations. Nevertheless, a consensus from several studies suggests that oligomeric protein assemblies physically disrupt cellular membranes affecting the calcium levels within the cell (Sanghera et al., 2008, Simoneau, et al., 2007, Zhang, et al., 2010). This may occur by the insertion of oligomers into the phospholipid membrane (Kayed et al., 2004) and, since the plasma membrane is accessible to both intracellular and extracellular proteins, perturbation of the membrane may provide a mechanistic link between protein misfolding diseases that are associated with either intra- or extracellular deposits. An alternative theory is that functional structures, composed of oligomeric proteins, form within the lipid bilayer. These structures appear to act as relatively porous ion channels, affecting the cellular membrane potential and ionic homeostasis, leading to apoptosis (Quist et al., 2005).

In contrast to oligomers, fibrillar aggregates of recPrP have shown variable toxicity *in vitro*: some researchers have found that fibrils are not toxic to cells (Simoneau, et al., 2007), suggesting that fibril formation *in vivo* is a protective mechanism or an "end-point" in the misfolding pathway (Caughey & Lansbury, 2003, Silveira et al., 2005). Conversely, other studies have shown fibrils to be just as toxic as oligomers (Novitskaya, et al., 2006), but since protein preparations are generally not extensively characterised prior to incubation with cells *in vitro,* it is possible that differences in protein structure may account for these inconsistencies. Although fibrils are typically perceived to be rather inert, it is also conceivable that smaller species could fragment from fibrillar aggregates, which may then possess the neurotoxic properties of oligomers (Tanaka et al., 2006). A study using Aβ fibrils showed that interaction of the fibrils with lipids led to fragmentation, forming oligomers which were highly toxic (Martins et al., 2008). Thus the toxicity of amyloid fibrils may be inversely proportional to their stability. However, Novitskaya *et al.* showed that fibrils composed of recPrP caused cells to aggregate and subsequently undergo apoptosis, an effect that wasn't seen for oligomers in the same experiments (Novitskaya, et al., 2006). This aggregation was reduced when PrP[C] was down-regulated, suggesting a role for PrP[C] in mediating toxicity. There have also been recent reports that PrP[C] is required for the toxicity exhibited by a range of molecular species (Resenberger et al., 2011a).

PrP[C] is expressed heavily at synapses and the misfolding process may initiate in and around the synaptic cleft. This localises all relevant molecular species in the compartment in which the first morphological changes are detected, but this is still someway short of proving that abnormal PrP is neurotoxic. Through ongoing studies in our laboratories, we are endeavouring to dissect the relationship between PrP[Sc], infectivity, neurotoxicity and mechanisms of neurodegeneration (Barron, et al., 2007, Bradford et al., 2009, Cancellotti et al., 2010, Cancellotti et al., 2005, Manson et al., 2001, Piccardo, et al., 2007, Tuzi et al., 2008, Tuzi et al., 2004). Through the use of several unique models of prion disease in mice, we are beginning to accumulate evidence suggesting that the levels of infectivity are not always dependent on the quantity of misfolded PrP present (Barron, et al., 2007, Piccardo, et al., 2007). In conjunction with studies on the neurotoxicity of misfolded recombinant prion proteins, this leads to the theory that specific subpopulations of PrP conformations represent

either the infectious or neurotoxic agents of TSEs (Weissmann, 1991). The role of PrP^C in neuronal toxicity is still controversial and prompts the question of whether a reduction in the levels of PrP^C on the cell surface, as it is converted into PrP^{Sc}, is a critical factor in prion disease-specific neurodegeneration.
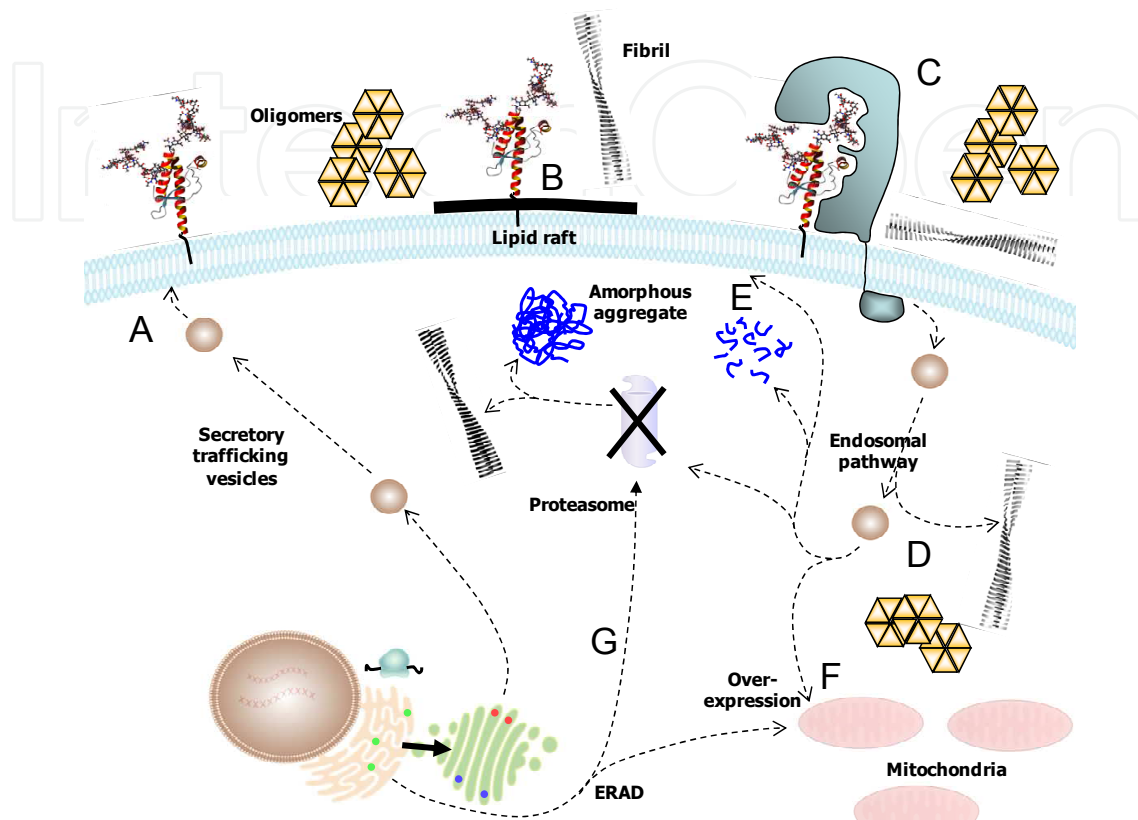

Fig. 2. Normal cell biology and putative misfolding pathways of PrP leading to toxicity. The prion protein is expressed in the secretory pathway and, after transiting the endoplasmic reticulum and Golgi apparatus, the protein is trafficked to the cell surface (A). Here it resides in specialised microdomains known as lipid rafts (B) but must move out of these domains to undergo endocytosis (C), presumably mediated by a cell surface receptor. After endocytosis PrP^C is routed on the endosomal pathway (D). Both the cell surface and the endosomal pathway have emerged as candidate locations for prion protein misfolding to intra- and/or extra-cellular oligomers or fibrils. After trafficking through endosomes, a proportion of the protein can be degraded, whilst some of it is routed back to the cell surface (E). Over-expression of PrP^C results in its localisation in mitochondria (F) whilst blockade of proteasome function leads to cytoplasmic accumulation (G). Both processes may follow retrograde transport of PrP from the ER by endoplasmic reticulum associated degradation (ERAD) processes.

3.2 Does loss of PrP^C function play a role in neurodegeneration?

The failure to infect prion protein knockout mice demonstrated conclusively that PrP^C is needed to sustain prion disease (Bueler, et al., 1993, Manson, et al., 1994b). These animals were also expected to inform on PrP function, but initial observations suggested that knockout mice developed normally (Bueler et al., 1992, Manson et al., 1994a). More in depth studies have highlighted a range of subtle and not so subtle alterations, including abnormal

circadian rhythms (Tobler et al., 1996), defects in long term potentiation (Curtis et al., 2003, Maglio et al., 2006) and abnormalities of mitochondrial numbers and morphologies (Miele et al., 2002). From additional studies, primarily in cells lacking PrP^C, there have been suggestions that PrP^C has roles in copper binding and trafficking (Brown et al., 1997), the response to reactive oxygen species (Brown et al., 1999), neuritogenesis (Graner et al., 2000, Lopes et al., 2005) and calcium homeostasis (Colling et al., 1996, Fuhrmann et al., 2006, Herms et al., 2001). A key recent finding in PrP^C knockout mice was defects in the maintenance of the myelin sheath surrounding peripheral nerves, a phenotype that appeared specifically to result from depletion of PrP^C from neurons (Bremer et al., 2010). Thus, prion protein knockout mice have a range of physiological phenotypes primarily related to neuronal functions and this has led to a consensus that PrP^C is a neuroprotective molecule, although it is not clear how, specifically, this neuroprotection is manifest (Resenberger et al., 2011b). A thorough review of prion protein function is beyond the scope of this chapter but, in the context of TSE disease, a key question is whether the loss of a neuroprotective function of PrP^C plays a role in neurodegenerative mechanisms. Some intriguing observations came from experiments in which tissue from PrP-expressing mice was grafted into the brains of PrP knockout mice. After intracerebral prion infection, the grafted tissue developed pathology typical of prion disease, including PrP^{Sc} deposition, neuronal loss and vacuolation (Brandner, et al., 1996). However, despite PrP^{Sc} spreading from the grafted tissue into the surrounding brain area, no loss of PrP-null neurons was observed. These data strongly suggest that PrP^{Sc} is not neurotoxic in the absence of PrP^C expression in neurons, results that were backed up by experiments in which PrP^C expression in neurons was conditionally turned off in mice during an ongoing prion infection (Mallucci et al., 2003). Further evidence comes from infection of PrP^C-GPI$^{-/-}$ mice (discussed further below), which do not express PrP^C on the surface of neurons or indeed any neural cells (Chesebro et al., 2005). In GPI$^{-/-}$ mice, significant levels of PrP^{Sc} accumulated during disease but neuronal loss was not observed. These lines of evidence suggest that PrP^C loss does not play a role in neurotoxicity and actually suggests the contrary – that normal neuronal PrP^C expression is required for neurotoxicity (Resenberger, et al., 2011a).

Contradictory evidence comes from studies in PrP^C-null mice transgenically expressing hamster PrP^C exclusively on astrocytes; these mice were capable of supporting hamster-passaged prion disease and developed clinical signs, indicating that neuronal PrP^C was not necessary for neuronal degeneration (Raeber et al., 1997). In the same studies, astrocytic hamster PrP^C was expressed in mice in addition to wild type murine PrP^C and these mice propagated hamster prion infectivity but did not develop disease, suggesting a role for mouse PrP^C in protecting neurons from the toxicity of PrP^{Sc}. There are also several studies demonstrating that the toxicity of prion-related polypeptides is independent of the expression of PrP^C on neurons. Hence, the role of PrP^C in neurotoxicity is not clear and further understandings of how misfolded proteins can lead to synaptic degeneration and/or neurodegeneration will require a closer relationship between *in vivo* and *in vitro* studies. It seems likely that the initiation and progression of pathology leading from synaptopathy to neuronal loss requires a combination of (i) interaction of PrP^{Sc} with the synaptic membrane/vesicle membranes (ii) ongoing PrP^{Sc} propagation (iii) loss of PrP^C function and (iv) extracellular toxic PrP^{Sc} deposits. Since misfolding of PrP is required for the pathology associated with TSE disease, understanding the factors that aid this process will also aid our understanding of neurotoxicity and neuronal loss.

4. The mechanisms of prion protein misfolding leading to neuronal loss

The prion protein is an obligatory component of TSE disease and its misfolding appears central to disease pathogenesis. Understanding how protein misfolding leads to pathology is of crucial importance but it is extremely challenging to study mechanistic aspects of protein folding and misfolding *in vivo*, hence *in vitro* studies have contributed almost all knowledge that currently exists in this area. This has involved solving and/or modelling structures of normal and aberrant forms of PrP and modelling the structural transition. The normal form of the protein has been investigated by use of recombinant prion proteins expressed in prokaryotic systems and refolded *in vitro*. The atomic level structures of such isoforms have been defined by both nuclear magnetic resonance (NMR) spectroscopy, for a range of different prion proteins e.g. (Calzolai et al., 2005, Christen et al., 2008, Gossert et al., 2005, Lysek et al., 2005, Perez et al., 2010, Wuthrich & Riek, 2001), and X-ray crystallography for sheep (Eghiaian et al., 2004, Haire et al., 2004) human (Antonyuk et al., 2009, Knaus et al., 2001, Lee et al., 2010) and rabbit proteins (Khan et al., 2010). These studies found that the C-terminal region of PrP has globular structure (depicted in Fig 3) and NMR investigations of native PrP^C purified from cattle brains confirmed these structural assignments (Hornemann et al., 2004).
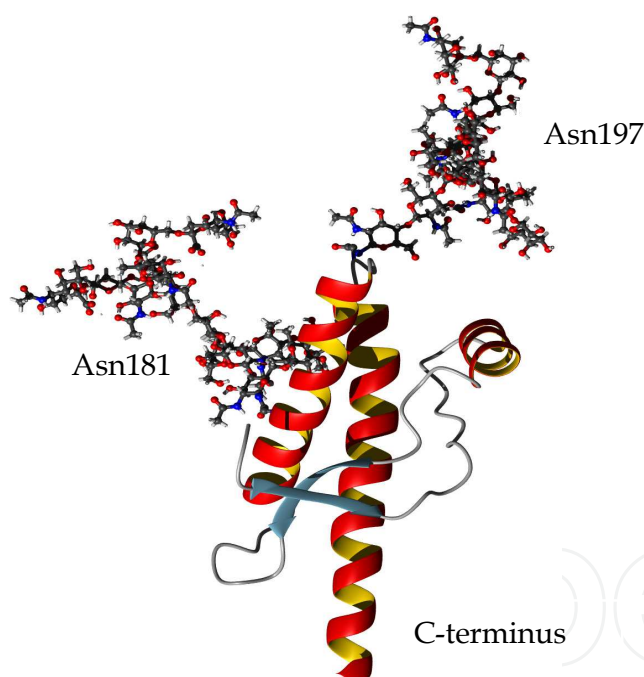

Fig. 3. The tertiary structure of PrP^C with two average sized N-linked glycans added at the two N-linked consensus sites (human numbering), to scale, demonstrating the contribution that these moieties make to the total volume of the prion protein

By contrast, the N-terminal region appears dynamically disordered. This domain incorporates 4-5 glycine-rich octapeptide repeats, which bind copper ions *in vitro* (Nadal et al., 2009, Pauly & Harris, 1998, Whittal et al., 2000, Wong et al., 2000a) and possibly *in vivo* (Brown, et al., 1997, Waggoner et al., 2000) and the region also mediates the binding of PrP^C to polyanionic compounds (Brimacombe et al., 1999). Although the N-terminal domain has been reported to be flexibly disordered, there have also been several reports of polyproline

II structure in this region (Blanch et al., 2004, Gill et al., 2000, Taubner et al., 2010). The N-terminal region is present in the majority of PrP[Sc] in diseased brains (Hope et al., 1986), but it seems to be dispensable for disease-specific misfolding, since transgenic mice expressing protein lacking the N-terminal domain are fully susceptible to disease (Fischer et al., 1996). The globular C-terminal region incorporates two consensus sites for N-linked glycosylation, a single disulphide bond and a glycosylphosphatidyl inositol (GPI) membrane anchor is appended to the extreme C-terminus. After conversion of PrP[C] to PrP[Sc], the C-terminal domain is resistant to protease digestion, indicating that it is this section of the protein that undergoes conformational change during prion protein misfolding.

By contrast to PrP[C], atomic level detail of PrP[Sc] tertiary structure is lacking, which is a result of the insolubility of PrP[Sc]-containing aggregates and the heterogeneity of morphologies of these aggregates. The structure of PrP[Sc] has been probed by use of several low resolution techniques and Fourier-transform infra red (FT-IR) spectroscopic analysis suggests that the transition from PrP[C] to PrP[Sc] is associated with a partial increase in β-sheet structure (Caughey et al., 1991). Initially it was proposed that the second and third α-helices are not misfolded and theoretical structures followed, the most detailed of which is based upon empirical structural investigations by electron crystallography (Govaerts et al., 2004, Wille et al., 2002). However, recent data from hydrogen/deuterium-exchange experiments in conjunction with mass spectrometry has cast doubt on the existence of α-helical sections in PrP[Sc] (Smirnovas et al., 2011). Instead, H/D exchange rates in PrP[Sc] appear consistent with formation of β-sheet across the entire C-terminal domain, a result that challenges conventional wisdom of PrP[Sc] structure. The problems associated with solving the structure of PrP[Sc] appear insurmountable, at least at the present time and we are more likely to derive useful information from reasonable models of PrP[Sc].

4.1 Misfolding of PrP can be modelled *in vitro*

The structural transition from PrP[C] to PrP[Sc] can be mimicked *in vitro* by a variety of techniques and this has allowed various determinants of protein misfolding to be investigated. By mixing together PrP[Sc] and recPrP expressed in mammalian cell lines to result in newly protease resistant PrP (PrP[Res]) the group of Byron Caughey showed that PrP[Sc] can auto-catalytically seed the conformational conversion of recPrP (Kocisko et al., 1994). This technique was termed the cell free conversion assay (CFCA) and it was subsequently shown to mimic many aspects of disease seen *in vivo*, including species barriers (Kirby et al., 2003, Kocisko et al., 1995) and the inhibitory effects of specific chemicals (Caughey et al., 1998, Demaimay et al., 2000, Demaimay et al., 1998). Quantifying conversion efficiency allows insights into mechanistic aspects of conversion: for example, there are two distinct phases of prion protein conversion – binding followed by conformational alteration (Horiuchi & Caughey, 1999) – and single amino acid substitutions were shown to dramatically affect the efficiency of conversion of the substrate (Bossers et al., 1997, Eiden et al., 2011, Kirby et al., 2010, Kirby et al., 2006). Furthermore, use of microsomes containing PrP[Sc] and PrP[C] in CFCA reactions indicated that the two proteins must be in the same vesicle for conversion to take place (Baron et al., 2002).

More recently, a second generation of *in vitro* prion misfolding assays has arisen, principally in response to the need for improved prion diagnostics. By the use of exogenous sources of energy to agitate the classical CFCA reaction, coupled with replenishment of the substrate,

conversion efficiencies can be dramatically enhanced. Conversion reactions driven by sonication or shaking have been developed and include methods known as protein misfolding cyclic amplification (PMCA) (Saa et al., 2006, Saborio et al., 2001), quaking induced conversion (QuIC) (Atarashi et al., 2011, Atarashi et al., 2008) and amyloid seeding assay (ASA) (Colby et al., 2007). The PMCA technique has also been shown to be capable of creating prion infectivity *de novo* from PrP^C substrate in the absence of a PrP^{Sc} seed (Castilla et al., 2005). The protocols for CFCA, PMCA or QuIC assays differ in detail but generic principles underlie all such assays, as depicted in figure 4. In all cases a catalytic seed of PrP^{Sc} causes misfolding of a substrate, and this phenomenon firmly establishes that auto-catalytic, templated misfolding is a generic process in prion diseases. In addition to sources of physical energy, believed to aid fragmentation of large fibrils thereby generating fresh seed, many of the prion amplification techniques also require facilitation with other factors to amplify both infectivity and misfolded protein (Deleault et al., 2007, Wang et al., 2010). In this context, it is notable that several techniques exist to misfold recPrP in the absence of a physiological seed. Pathways leading to fibrils (Baskakov et al., 2002, Stohr et al., 2011) or oligomers (Rezaei, 2008, Tahiri-Alaoui et al., 2004, Tahiri-Alaoui et al., 2006) have been described, where misfolding is promoted by partially denaturing conditions. These processes occur comparatively rapidly and generally do not replicate features of disease, such as species barriers (Makarava et al., 2007) or polymorphic control of susceptibility (Baskakov et al., 2005, Kirby, et al., 2010), and also do not appear to generate *bona fide* prion infectivity (Legname et al., 2004, Makarava et al., 2010). These lines of evidence argue for a role for molecular cofactors in disease-specific prion protein misfolding (Birkmann & Riesner, 2008, Gill et al., 2010, Graham et al., 2010). Identifying these co-factors *in vivo* will allow significant progress in the prevention of disease transmission.
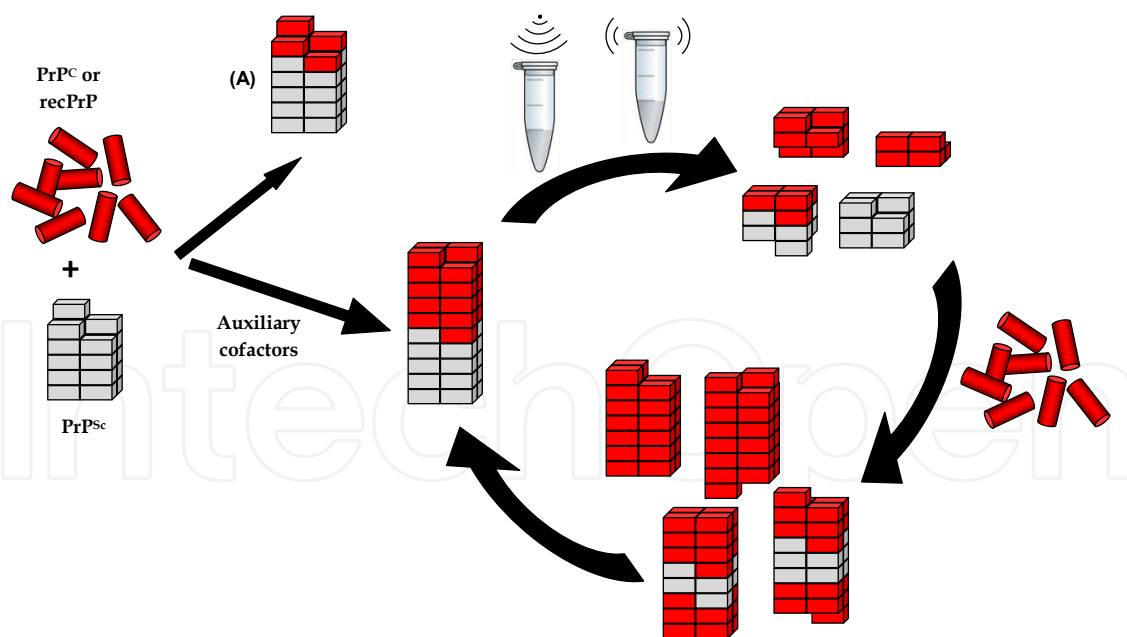

Fig. 4. Schematic pathways for seeded conversion of normal PrP to a protease-resistant isoform. In the absence of auxiliary cofactors, conversion is inefficient and only a small amount of available substrate is converted (A). This pathway is exemplified by the classic CFCA. By addition of auxiliary cofactors, conversion efficiency can be improved and periodic shaking (QuIC) or sonication (PMCA), coupled with replenishment of substrate, allows cyclic conversion leading to amplification of PrP^{Res}

4.2 Factors contributing to prion protein misfolding

Dramatic breakthroughs in the search for determinants of the prion protein misfolding process have been made in recent years. Deleault *et al* used the PMCA technique to amplify PrP^C that had been highly purified from brain tissue to which polyanionic species (RNA or glycosaminoglycans) were added. This mixture was sufficient to allow amplification of abnormal PrP when seeded with PrP^{Sc}, but also allowed the generation of abnormal PrP *de novo* in the absence of a catalytic seed. Crucially, the newly-synthesised abnormal PrP was shown to cause a TSE-like disease after inoculation to wild type animals. These data imply that purified PrP^C (along with lipids that co-purified with the protein) in addition to a polyanionic cofactor are the minimal requirements for creation of prion infectivity (Deleault, et al., 2007). Various researchers have since replicated or extended this work (Barria et al., 2009, Edgeworth et al., 2010, Weber et al., 2007), culminating in the publication of a study describing prion infectivity, created *de novo*, from bacterially-expressed recPrP supplemented with just synthetic lipid and total RNA extracted from murine liver (Wang, et al., 2010). What are the identities of molecules playing the roles of cofactors *in vivo*?

One approach to determine *in vivo* cofactors is to investigate the aggregates present in prion-infected animals for molecules that may have played a role in their formation. Other than PrP, various proteinaceous molecules appear specifically enriched in infectious prion fibrils (Giorgi et al., 2009, Moore et al., 2010, Petrakis et al., 2009) and recent data from our laboratory suggest that at least one such protein can enhance prion protein conversion efficiency (Graham et al., 2011). The most likely places for PrP^C to encounter PrP^{Sc} and for conversion to take place are on the cell surface or within the endocytic pathway and it would appear reasonable to expect cofactors to reside in these locations. Results from experiments in cell lines supporting either location as a site for conversion have been published (Borchelt et al., 1992, Hooper, 2011). Recent data from our laboratories (Graham, et al., 2010, Graham, et al., 2011) and others (Abid et al., 2010), suggest that the plasma membrane is a more likely source of cofactors modulating prion protein misfolding. It is plausible that specific compositions of lipid can modulate prion protein structure thereby creating conditions for strain specific misfolding. Misfolding in or around the plasma membrane would facilitate toxic mechanisms that involve disturbances in membrane permeability. There are also various properties intrinsic to the prion protein that exert an influence on misfolding and which therefore may impact on neuronal toxicity of the resulting aggregates. Amino acid substitutions in the prion protein affect susceptibility of animals to prion disease and mutations in the human *PRNP* gene (encoding the prion protein) appear to be a direct cause of familial prion diseases. In general, those amino acid substitutions associated with resistance to prion disease in animals appear to decrease the stability of recombinant prion proteins *in vitro* (Bujdoso et al., 2005, Kirby, et al., 2010, Paludi et al., 2007, Thackray et al., 2004) potentially leading to differing levels of cellular toxicity. By contrast, there is conflicting data on the ability of mutations associated with human familial disease to affect the structure and stability of PrP^C (e.g. (Apetri et al., 2004, Bae et al., 2009, Inouye et al., 2000, Rossetti et al., 2011, van der Kamp & Daggett, 2010, Vanik & Surewicz, 2002, Yin et al., 2007)) and there is a lack of clear data suggesting that human mutations confer increased neurotoxicity upon misfolded PrP. It seems likely that the effects of individual amino acid changes depend on the specific substitution as well as the position within the sequence of PrP^C and potentially the species that the amino acid change is in.

PrP[C] undergoes various post translational modifications *in vivo* and many have been investigated for their impact on prion protein misfolding. In transgenic mice that express prion protein lacking the C-terminal signal sequence, the GPI anchor is not attached (GPI^{-/-}) and this results in secretion of PrP[C] into the extracellular milieu. When GPI^{-/-} mice are infected with a prion disease there is dramatic accumulation of large amyloid plaques composed of anchorless PrP but no evidence of neurodegeneration (Chesebro, et al., 2005). The reasons for this are unclear but presumably result from the lack of association of PrP[C] with the plasma membrane (Caughey et al., 2009), however, preventing GPI anchor addition also inhibits glycosylation of PrP[C] and this may be a compounding factor in the lack of pathology/disease. Nevertheless, as mentioned earlier, these results further indicate that large aggregates composed of prion protein are not neurotoxic *per se*.

N-linked glycosylation of PrP[C] occurs at two sites in the C-terminal region of the protein (Rudd et al., 2002) and a variety of techniques have been used to study the effect of glycosylation on prion protein misfolding. *In vitro* studies suggest that glycosylation of PrP[C] affects its interaction with PrP[Sc] (Priola & Lawson, 2001), but that glycosylation is not required for strain properties (Nishina et al., 2006, Piro et al., 2009). Initial reports from studies in cell lines suggested that removing prion glycosylation produced spontaneously misfolded protein (Lehmann & Harris, 1997), however, this may have been a result of over-expression, since more recent studies have shown that blocking glycosylation of endogenously expressed PrP[C] does not produce this phenotype (Cancellotti, et al., 2005). In some cases, studies have been hampered by the folding and trafficking abnormalities that can occur when PrP[C] is expressed without glycosylation (Cancellotti, et al., 2005, DeArmond et al., 1997) depending on the specific mutations used to prevent glycosylation (Capellari et al., 2000, Ikeda et al., 2008, Salamat et al., 2011, Wong et al., 2000b). Neuendorf *et al* selected deglycosylating mutations that retained authentic PrP[C] cellular trafficking and mice in which these proteins were over-expressed were susceptible to both scrapie and BSE (Neuendorf et al., 2004). However, in some cases, incubation times were shorter than with wild type mice, which is probably an artefact of over-expression. In our laboratories we have produced gene-targeted mice lacking prion protein glycosylation (Cancellotti, et al., 2005) and analysis of these mice confirm that glycosylation is important for efficient trafficking of PrP[C], but that glycosylation is not always required to sustain prion infection after intracranial inoculation (Tuzi, et al., 2008). Intra-cranial infection of these mice with multiple prion strains indicates dramatically different requirements for occupation of each of the glycosylation sites of host PrP for infection. However, since disease outcomes are significantly modulated following peripheral infection of glycosylation-deficient, gene-targeted mice, our data also suggest that glycosylation of PrP is important for either peripheral replication of PrP[Sc] or for trafficking of the infection to the CNS (Cancellotti, et al., 2010). The glycans present at either site are highly heterogeneous (Ritchie et al., 2002, Rudd et al., 1999, Stimson et al., 1999); at least 60 different glycan moieties can be present on the protein and genetic removal of glycosylation does not distinguish between individual glycan structures. Thus, it is unclear whether any individual carbohydrate chains render the prion protein particularly susceptible to misfolding.

In summary, although we know the structure of PrP[C] to atomic resolution and we can model the conversion to PrP[Sc] *in vitro,* the details of how this process takes place *in vivo* are still unknown. Although various factors are known to affect the way that PrP[C] may misfold,

the only factor absolutely known to direct this process is exogenous PrP^{Sc}. It is assumed that the PrP^{Sc} catalysed misfolding of PrP^C results in a species that is neurotoxic, but it remains possible that loss of PrP^C is an important process in mediating neurotoxicity. Once neurotoxicity results, it appears clear that synaptic dysfunction is one of the first pathological alterations that can be detected. Approaches that integrate studies of protein misfolding, *in vitro* toxicity and *in vivo* toxicity are required to allow us to address the many unknowns of neuronal loss in prion disease

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6. References

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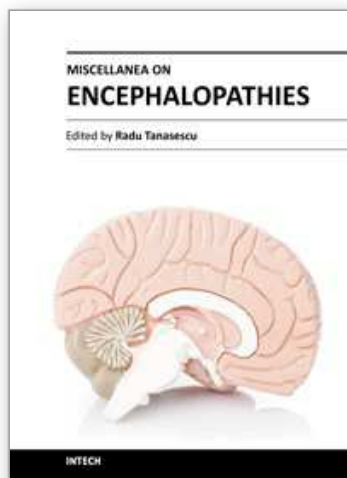
Miscellanea on Encephalopathies

Edited by Dr. Radu Tanasescu

The book project "Miscellanea on Encephalopathies" aims to cover some of the important aspects of infectious-related encephalopathies, post-transplantation and drug-induced encephalopathies, by transmitting valuable information filtered through the real life clinical and research experience of the authors.

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