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MINI-REVIEW

Mayhem of the multiple mechanisms: modelling neurodegeneration in prion disease

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Abstract

This review examines recent attempts to advance the understanding of the mechanism by which neurones die in prion disease. Prion diseases or transmissible spongiform encephalopathies are characterized by the conversion of a normal glycoprotein, the prion protein, to a protease-resistant form that is suggested to be both the infectious agent and the cause of the rapid neurodegeneration in the disease. Death of the patient results from this widespread neuronal loss. Thus understanding the mechanism by which the abnormal form of the prion protein causes neuronal death might lead to treatments that would prevent the life-threatening nature of these diseases.

Keywords: apoptosis, copper, microglia, oxidative stress, prion, toxicity.

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In the 1990s reviews of research in the field of prion diseases rarely discussed the mechanism of neurodegeneration (Prusiner and DeArmond 1994). At first this would appear strange because the fundamental threat that prion diseases present is that they are fatal neurodegenerative conditions. However, the prion protein (PrP^c) was not sequenced until 1985 (Oesch *et al*. 1985; Basler *et al*. 1986). The main bone of contention in the field for many years was the suggestion made by Stanley Prusiner that the abnormal isoform of this protein (PrP^{Sc}) which could be isolated from the brain of mice infected with mouse passaged scrapie (Bolton *et al*. 1982), was the sole agent of infection (Prusiner 1982). All prion diseases, including Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy (BSE), scrapie in sheep and chronic wasting disease (CWD) in deer, can be transmitted from individual to individual or even across species by injecting a brain extract from an infected individual into the brain of another. Prion diseases are not naturally spread this way, and indeed the majority of prion diseases including CJD and scrapie are sporadic occurring with no evidence of transmission of any kind. Nevertheless, research on the mechanism by which prion disease can be transmitted experimentally has continued with many people still rejecting the prion hypothesis, despite Stanley Prusiner winning the Nobel Prize for his attempts to prove this theory (Prusiner 1998). The most convincing evidence for the role of the prion protein in prion disease transmission comes from work with mice in which PrP^c expression was ablated or knocked out (Büeler *et al*. 1992). In the absence of PrP^c expression mice are completely resistant to scrapie infection (Büeler *et al*. 1993). The reason for this is that there is no PrP^c to be converted to PrP^{Sc}. Clearly, understanding the conversion of PrP^c to PrP^{Sc}, either by exogenous PrP^{Sc} or by some other mechanism, is central to understanding these diseases. Although exogenous PrP^{Sc} is an important consideration for investigations of the conversion process, sporadic diseases where there is no exogenous PrP^{Sc} introduced will not be explained by this. The first study to advance an alternative suggested that metal substitution or loss of binding at the copper binding site of PrP^c might be sufficient to generate an abnormal form of PrP^c (Brown *et al*. 2000; Brown 2001a).

Studies of neurodegeneration in prion disease advanced little during this time. The first studies of neuronal death used cell culture models (Müller *et al*. 1993; Forloni *et al*. 1993). This approach has remained the preferred model for the majority of investigators. Unfortunately, there has been little crossover of ideas from the cell culture models to work with animals, or at least little has been verified in animal models to

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Abbreviations used: CJD, Creutzfeldt–Jakob disease; GPI, glycosylphosphatidylinositol; PrP^c, prion protein; PrP^{Sc}, abnormal isoform of prion protein.

date. Müller *et al*. (1993) showed that PrP^{Sc} is toxic to cultured cells. This study was also the first to suggest that the mechanism of cell death involved calcium entry via NMDA receptors. The difficulty in isolating PrP^{Sc} and the general inability to prove that it is pure has lead investigators to use synthetic peptides. Forloni *et al*. (1993) identified a 21 amino acid peptide (PrP106–126) that appeared to represent the neurotoxic core of the protein. Since then virtually every group studying neuronal death in prion diseases has either used this peptide or sequences equivalent to it. Thus apart from one very poorly controlled study (Kunz *et al*. 1999; see Brown 1999) the neurotoxicity of this peptide has been heavily and thoroughly reproduced. Indeed, the main concern that this peptide is not neurotoxic *in vivo* has also been shown to be false following a study showing that the PrP106–126 peptide is toxic to cells in the retina of rats (Ettaiche *et al*. 2000). Additionally, the 'mini-prion' a 106 amino residue PrP expressed in transgenic mice retains the 106–126 region of the protein (Supattapone *et al*. 1999). This PrP fragment is sufficient to induce prion disease and neuronal death *in vivo*. My own research of the PrP106–126 peptide has been quite extensive, and has been reviewed recently a number of times (Brown 2001b,c).

In parallel with PrP106–126, other short peptides that were based on the sequence of PrP^{Sc} have also been studied. However, the toxicity of other peptides was either found to be much less than with PrP106–126 (Brown 2000a) or dependent on mutations involved in inherited prion disease (Forloni *et al*. 1999). In some cases different groups have reported these peptides to be non-toxic (Forloni *et al*. 1993), and others have reported that they are toxic (Pillot *et al*. 2000; Rymer and Good 2000). In absence of consistent findings most interest has remained with PrP106–126.

PrP106–126 has many physiochemical qualities similar to those of PrP^{Sc}. These include protease resistance, high β-sheet content and the propensity to aggregate into fibrils (Forloni *et al*. 1993; Tagliavini *et al*. 1993; De Gioia *et al*. 1994; Salmona *et al*. 1999). These findings show that in many ways PrP106–126 is a good mimic of PrP^{Sc}. Such qualities are clearly important considerations when considering the neurotoxic nature of either PrP^{Sc} or PrP106–126. Aggregation has been suggested to be important to the toxicity of PrP106–126. However, it has also been shown that soluble non-aggregated peptide is also important to toxicity (Brown *et al*. 1998a). However, others have argued that fabrillogenesis is totally unnecessary for toxicity (Salmona *et al*. 1999). Thus, although the physical state of PrP106–126 can mimic PrP^{Sc} it is not clear whether this has relevance to neurotoxicity.

Research using PrP106–126 continues to be an expanding field with many new groups proposing new insights or new mechanisms for the action of this peptide. In the last year alone there have been more than 25 publications on the action of PrP106–126 (e.g. Haïk *et al*. 2000; Rymer and Good 2000; Bate *et al*. 2001; Brown 2001b,c; Deli *et al*. 2001; Della-Bianca *et al*. 2001; Fabrizi *et al*. 2001; Gu *et al*. 2001; Kourie *et al*. 2001; Le *et al*. 2001; O'Donovan *et al*. 2001; Stewart *et al*. 2001; White *et al*. 2001). These different mechanisms suggest a variety of factors that might be involved. They range from interactions between PrP106–126 at the membrane, with proteins such as the p75 NGF receptor (Della-Bianca *et al*. 2001), formyl peptide receptor-like 1 (Le *et al*. 2001), L-type calcium channels (Florio *et al*. 1996), extendin (Martins *et al*. 1997; Lopes *et al*. 2001), laminin (Graner *et al*. 2000), vitronectin (Hajj *et al*. 2001), PrP itself (Brown 2000b), or other proteins such as DNA (Nandi 1997), tubulin (Brown *et al*. 1998b) and Bcl-2 (Kurschner and Morgan 1995). Additionally, it has been suggested that the peptide binds to copper (Jobling *et al*. 2001; Brown 2000a) as has the whole protein (reviewed in Brown 2001a). Indeed the list of proposed binding partners for PrP (not just the peptide) has been growing and includes dystrophin (Keshet *et al*. 2000), the laminin-receptor (Rieger *et al*. 1997) and others (Yehiely *et al*. 1997). Clearly, this expanding list requires rationalization. It is likely that many of these interactions will prove to be artefacts that occur in the test tube but have no physiological relevance. In particular, the suggested interaction between Bcl-2 family members and PrP is likely to be a pure artefact. Many proteins bind to Bcl-2 family members simply as a result of particular detergent conditions used during the isolation of protein extracts (Hsu and Youle 1997). Unfortunately, the more binding partners that are suggested for the protein or the peptide, the less credibility any such proposed partner has either in terms of the function of the protein or in terms of activation of cell death pathways.

Other proposed mechanisms suggest that PrP106–126 can either interact directly with the membrane (Rymer and Good 2000) and generate a transmembrane channel (Kourie and Culverson 2000; Kourie *et al*. 2001) or can alter membrane fluidity (Salmona *et al*. 1997). These effects are not irreconcilable with other effects of the peptide. Indeed, given that PrP106–126 is quite hydrophobic it is likely that the peptide will readily enter the lipid bilayer. This hydophobicity might explain why it readily binds to other proteins and explain the multiple binding partners mentioned above. PrP^{c} is normally a glycosylphosphatidylinositol (GPI)-anchored protein tethered to the cell surface without passing through it (Stahl *et al*. 1990). Studies with full-length proteins have suggested that under abnormal conditions PrP can also become transferred into the membrane because of a potential stop transfer element (STE). The domain which is then transmembrane is the same region that contains the PrP106–126 sequence (Hegde *et al*. 1998). Mutations in the *prnp* gene have been shown to alter the proportion of PrP that can be inserted in this transmembrane form. Unfortunately, the relevance this has for disease remains disputed with reports suggesting that at best only a few point mutations that are linked to inherited forms of prion disease would lead to

transmembrane PrP (Stewart and Harris 2000). Additional claims that transmembrane PrP accounts for neurodegeneration in prion disease independent of association with inherited mutations in the gene are still speculation (Hegde *et al*. 1999). A recent paper has suggested that interaction between PrP106–126 and PrP^{c} expressed by neuronal-like tumour cells over a period of four months (!) results in a form of truncated PrP that could become transmembrane (Gu *et al*. 2001). However, a previous study (Brown 2000b) has shown that such truncated PrP accumulates in aggregates of PrP106–126 (after one day of exposure), which can be readily separated from cells thus suggesting that this truncated PrP is not a transmembrane form. Another report shows that the same region of PrP proposed by Hegde *et al*. (1999) to be transmembrane is neurotoxic (Haïk *et al*. 2000). However, this peptide is virtually identical in sequence to PrP106–126 and there is little to suggest that the majority of peptide added by the authors to their cultures actually becomes inserted in the membrane. Indeed these peptides aggregate rapidly when added to a salt solution and float around in clumps in the culture medium. Therefore neither of these studies could be said to provide evidence that transmembrane PrP is necessary for neurotoxicity.

Recently, some researchers have decided to investigate further. Instead of just studying the direct effects of the peptide on cells as if they were a mysterious black box, they have chosen to look inside to see which effects might actually proceed cell death. As has been mentioned already, a number of studies have looked at calcium changes but there seems to be some degree of lack of certainty: reports suggest that calcium entry increases calcium release (Müller *et al*. 1993; Whatley *et al*. 1995) but others say that PrP106–126 reduces calcium release (Thellung *et al*. 2000). Although it has been suggested that PrP can interact with Bax, another study suggests that PrP106–126 causes mitochondrial depolarization (O'Donavan *et al*. 2001). Further downstream in the cell-death pathway are the executors of cell suicide, the caspases. There is now evidence that some of the caspases become activated by PrP106–126 (White *et al*. 2001). This finding is not unexpected as caspases are commonly involved in cell death. Other findings from the same group suggest that production of toxic arachidonic acid metabolic products might be involved in cell death (Stewart *et al*. 2001). Another more common theme is that of changes in antioxidant proteins that would otherwise protect the neurone from its oxidatively stressing environment (Brown *et al*. 1996; Perovic *et al*. 1997; Rizzardini *et al*. 1997).

How does one begin to distinguish the true mechanism of neurodegeneration in prion disease from all of these possible disparate observations? The first way is to identify those changes that are reproducible between different research groups and the second way is to show that the mechanism is actually occurring in an animal with prion disease. At present only a few such findings have been confirmed at either level.

PrP-knockout mice are resistant to prion infection (Büeler *et al*. 1993). As these mice cannot generate PrP^{Sc} it is difficult to determine whether PrP^{Sc} is toxic in the absence of neuronal expression of PrP^{c} *in vivo*. However, an ingenious transplantation experiment showed that PrP^{c} expression is necessary for the toxicity of PrP^{Sc} (Brandner *et al*. 1996). Transplantation of embryonic tissue from a mouse over-expressing PrP^{c} was made into a PrP-knockout mouse brain. The transplanted tissue was then infected with mouse scrapie. This transplanted tissue generated PrP^{Sc} and showed signs of neurodegeneration and gliosis, whereas the PrP-knockout brain surrounding it remained untouched by neurodegeneration. PrP^{Sc} accumulated in the PrP-knockout mice brains but this did not cause neurodegeneration. These results show that neurones must express PrP^{c} in order to be killed by the agent of neurodegeneration in prion disease. Assuming that this agent is solely PrP^{Sc} then PrP^{Sc} requires neuronal expression of PrP^{c} to be neurotoxic. PrP106–126 also requires neuronal expression of PrP^{c} to be neurotoxic as first shown in 1994 (Brown *et al*. 1994). This finding in culture has also been shown for PrP^{Sc} itself (Giese *et al*. 1998) and has been verified by other groups (Jobling *et al*. 1999). As this finding has been confirmed *in vivo*, *in vitro* and by a number of groups it can therefore be assumed that neuronal PrP^{c} expression is the first essential component of the neurotoxic mechanism of PrP106–126. Thus any group suggesting that they have found the mechanism of action of PrP106–126 or PrP^{Sc} should confirm that their mechanism does not occur in PrP-knockout neurones or in the absence of PrP^{c} expression.

Oxidative stress has been shown to be a hallmark of prion diseases (Guentchev *et al*. 2000; Wong *et al*. 2001) as with many neurodegenerative diseases. Numerous groups have shown that PrP106–126 causes oxidative stress, disturbs the expression of antioxidant proteins or that the toxicity of PrP106–126 can be inhibited by antioxidants (Brown *et al*. 1996; Perovic *et al*. 1997). Cultures infected with PrP^{Sc} are more susceptible to the toxicity of reactive oxygen species (Milhavet *et al*. 2000). There is currently no evidence that antioxidants can inhibit neuronal death *in vivo*. However, it is clear that oxidative damage is involved in the mechanism of PrP106–126 in the culture system. Therefore this is a logical next component of the mechanism of action of the peptide. There is little doubt that PrP106–126 causes cell death via an apoptotic mechanism, and as the apoptosis of neurones involves such changes as caspase activation (White *et al*. 2001), mitochondrial depolarization (O'Donavan *et al*. 2001) and enhanced calcium entry through either NMDA receptors (Müller *et al*. 1993) or L-type voltage gated calcium channels (Brown *et al*. 1997) then it is logical to assume that any or all of these changes might be induced by the peptide or PrP^{Sc}. However, none of these changes are specific and so can be safely termed as 'downstream' events that occur as a result of the triggering of apoptosis. Therefore

although they are likely to be parts of the mechanism they are not specific to prion disease.

As has been stated above, PrP^{Sc}-infected cells are more sensitive to oxidative stress applied to the culture. This suggests an indirect effect. Other studies have shown that PrP106–126 reduces neuronal 'resistance to oxidative stress' (Brown *et al*. 1996). Thus the implication is that the toxic effect is indirect as it requires oxidative stress to come from outside the cell in order to initiate apoptosis. A number of authors have stated that the peptide has a direct effect and does not require these indirect stresses. However, such statements are rather ignorant as a compromised antioxidant defence on its own will not kill a cell, and many culture systems might provide sufficient oxidative stress without oxidative substances being added to the culture. Glutamine, a common additive to culture medium, is rapidly converted to glutamate. The levels of glutamate in most culture systems are sufficient to activate a certain level of spontaneous apoptosis (mediated by intracellular superoxide production) in cells expressing NMDA receptors. Thus those researchers who claim a 'direct' effect that is independent of oxidative damage must show that their mechanism cannot be inhibited by antioxidants applied to their cultures. If antioxidants are effective then it is clear that an 'indirect component' is involved.

Nevertheless, a direct component is necessary for the toxicity of PrP106–126 as PrP^{c} expression by neurones is necessary for the toxicity to those neurones (Brown *et al*. 1994). It is this complexity that often frustrates those trying to understand the mechanism of PrP106–126. Many assume that a toxic mechanism must be X and does Y. However, it appears that in prion disease it is more accurate to describe the process as X does Y when Z has occurred. In this case X is the increased production of an oxidative substance caused by PrP106–126, Y is neuronal death and Z represents reduced neuronal resistance to oxidative stress caused by PrP106–126. Therefore those considering the neurotoxic mechanism of this peptide must consider both a direct and an indirect component to the mechanism. It is not sufficient to simply assume that because one adds a peptide to an apparently homogeneous population of cells and the cells die that this is caused by a direct effect alone.

Microglial cells are activated by either PrP106–126 or PrP^{Sc}. This finding has now also been repeated by a number of groups (Brown *et al*. 1996; Giese *et al*. 1998; Combs *et al*. 1999; Fabrizi *et al*. 2001). Activated microglia release toxic substances such as superoxide or tumour necrosis factor (TNF)-α, which might contribute to indirect toxic effects. In particular microglia were suggested to be a component of the first mechanism for PrP106–126 toxicity proposed (Brown *et al*. 1996). It should be noted that when this model was first proposed it was shown that microglia per se are *NOT* essential, but that a source of oxidative stress (such as that produced by microglia) was. Microglia also enhance the toxicity of PrP^{Sc} (Giese *et al*. 1998) and microglia are activated before the onset of neurodegeneration in mouse scrapie (Williams *et al*. 1994; Giese *et al*. 1998). Since these first reports, the finding that microglia can enhance the toxicity of both PrP^{Sc} and PrP106–126 has been independently confirmed (Bate *et al*. 2001). This implies that this is a reproducible effect confirming that an indirect effect of substances released by microglia can contribute to the neurotoxic mechanism of prion disease.

Thus, in summary, those components of the neurotoxic mechanism of PrP106–126 or PrP^{Sc} that have been confirmed and reproduced and which should be considered by anyone investigating this topic are: (i) the peptide or PrP^{Sc} is not toxic in the absence of PrP^{c} expression; (ii) the mechanism involves both direct and indirect effects; (iii) the indirect effects include the generation of oxidative stess; (iv) the direct effects result in reduced resistance to oxidative stress (see Fig. 1). In the context of these confirmed findings the plethora of new ideas about the mechanism need to be carefully assessed and equated with this stand-point. It is to some degree absurd for researchers to ignore the literature and to suggest that no model of the mechanism currently exists. Thus, the onus is on the proposers of these new models to disprove this model first. However, clearly there are assumptions made in this model that might be inadequate to describe the mechanism of neurodegeneration in prion disease.

One of the big assumptions in all of the foregoing discussion is that the toxicity of PrP106–126 is all that there is to the toxicity of the prion protein. An important study based on an animal model definitely shows that this is not the case. Mice expressing a truncated form of PrP starting at either amino-residue 121 or 136 in the mouse sequence on a PrP-knockout background (i.e. no wild-type PrP is expressed in these mice) show rapid neurodegeneration soon after birth (Shmerling *et al*. 1998). The PrP expressed by these mice lacks the PrP106–126 region but there is nevertheless clear evidence that the C-terminal portion of PrP is neurotoxic. My group has recently shown that the prion protein contains a second toxic domain. This domain stretching around amino residue 147–220 appears to be more toxic to neurones than PrP106–126 (Daniels *et al*. 2001). This C-terminal fragment is more toxic to neurones lacking expression of PrP^{c}. This fits with the observations of Shmerling *et al*. (1998) who suggested that full-length PrP^{c} inhibited the toxicity of PrP121–231. Experiments with full-length recombinant PrP^{c} in culture also confirm this to be the case (Daniels *et al*. 2001). A more surprising finding, however, is that the neurotoxic peptide PrP106–126 also inhibits the toxicity of PrP121–231 (Daniels *et al*. 2001). Thus PrP not only has two potentially neurotoxic domains but it is highly likely that in an intact molecule these two domains neutralize each other in terms of this potential neurotoxicity. Current studies of neurodegeneration in prion disease do not take these observations into account. The toxicity of PrP121–231 is not

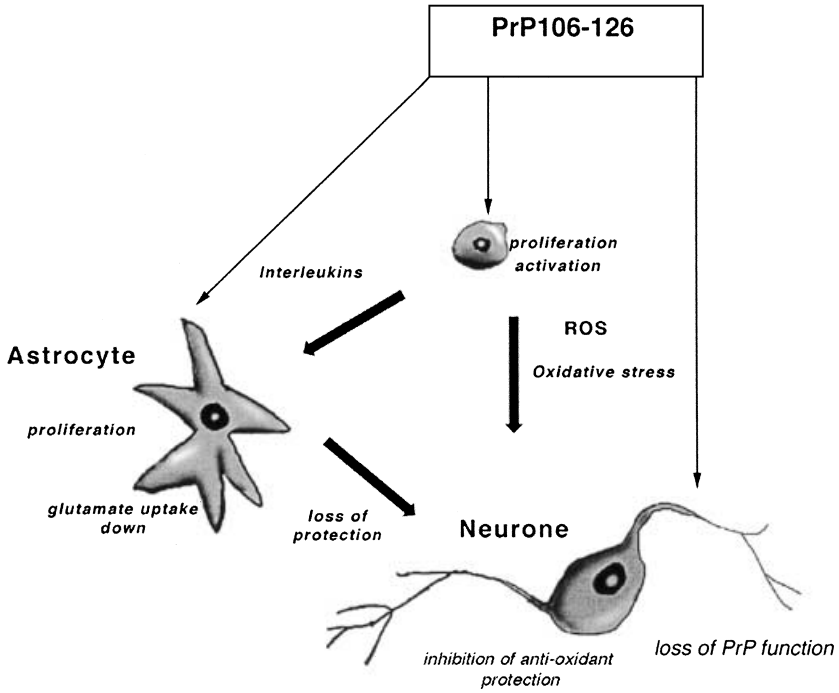

Fig. 1 Schematic representation of the mechanism of toxicity of the PrP106–126 peptide.

sufficient or adequate to explain the toxicity of PrP^{Sc}. As pointed out earlier PrP^{Sc} is not toxic to PrP-knockout neurones. Nevertheless, the cell death that occurs in prion diseases *in vivo* might not be explained by results from *in vitro* studies of PrP106–126 or PrP^{Sc} alone. These recent studies may change the focus of research away from PrP106–126. What is yet to be determined is the extent of which the new toxic domain contributes to neurodegeneration *in vivo*. It is quite possible that changes in the secondary structure of PrP^{c} when converted to PrP^{Sc} cause the two toxic domains to be unmasked. It is quite possible that in the presence of functional PrP^{c} the more C-terminal toxic domain is not involved in mediating cell death. Much further research is needed to unravel the importance of these new observations.

These discussions suggest that although many new findings have emerged about the nature of neurodegeneration in prion disease they have posed more questions than they have provided answers. However, the myriad of new ideas and new proposed mechanisms have thrown the field into a perhaps cathartically necessary 'mayhem.' However, from among these many investigations certain clear paths to the truth stand out. If researchers focus on these clear paths then it is likely that important advances will be made. However, it is possibly necessary that those research groups dedicated to reaching these advances communicate and interact more. If more groups continue to 'show for the first' another possible mechanism that they propose to be 'the answer' then we are likely to lose sight of the important question that remains to be answered: 'How can neurodegeneration in prion disease be prevented?' The future of research into the field of prion diseases has several clear targets. Meeting these are important for dealing with the threat of variant Creutzfeldt–Jakob disease. These targets include: (i) diagnosing prion diseases before clinical signs resulting from neuronal loss occur; (ii) inhibiting neuronal loss in prion disease; (iii) reversing clinical signs in prion disease. Clearly, these targets are all dependent on a better understanding of changes in neurones caused by abnormal forms of PrP. The *in vitro* models that have been used for some years to study pathogenesis of prion disease have now been shown to be relevant *in vivo* at a basic level. It is now time to reassess what has been learned *in vitro* and try to see if this information will bring us closer to dealing with the targets indicated here.

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