CNS pathogenesis of prion diseases

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Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases, clinically characterised by cognitive decline, paralleled by severe damage to the central nervous system. Prion diseases have attracted a broad interest because of their unique mechanisms of replication and propagation; however, the underlying pathogenic mechanisms are still highly speculative. In this review, current knowledge about the pathogenesis of prion diseases in the CNS will be highlighted and the most revealing animal models will be discussed, with future perspectives to address immediate questions about the pathogenesis.

Historically, prion diseases have been characterised neuropathologically by neuronal vacuolation (spongiosis), brisk reactive proliferation of astrocytes and microglia, and by the deposition of amyloid plaques. These plaques have recently been identified as aggregated forms of the pathological form of PrP and can be readily detected by routine immunohistochemistry. Many neuropathological studies have been carried out to characterise meticulously and categorise patterns and types of prion protein deposits in various areas of the brain, and to correlate these with distinct neurological signs in patients succumbing to disease. Unfortunately, however, this has often led to limited advances in the understanding of TSE pathogenesis.

As in other neurodegenerative diseases, such as Alzheimer’s disease (amyloid precursor protein and tau), Parkinson’s disease (α-synuclein) or Huntington’s disease (huntingtin), faulty processing of proteins, may result in the deposition of an abnormally folded, poorly metabolised aggregate which eventually is deposited intra-or extracellularly. So far, the pathogenesis of all these neurodegenerative diseases has appeared straightforward and the discovery of the pathomechanisms involved in the abnormal processing of cellular proteins may have raised premature expectations that effective therapeutic approaches may be within reach.

The mechanism of neurodegeneration in prion diseases is still poorly understood, and not much is known about the mechanism by which prions actually impair neuronal function and cause cell death, despite important contributions from animal models to (i) verify the protein-only
hypothesis\(^1,2\); (ii) determine the molecular basis of the species barrier\(^3\textendash6\); or (iii) characterise the entry route and neuro-invasion of prions\(^7\textendash9\).

**The pathogenesis of prion diseases**

Important supporting evidence for the protein-only hypothesis was the generation of mice with targeted disruption of the *Prnp* gene\(^1,10,11\). Although it was proposed that the ubiquitously expressed neuronal protein PrP\(^C\) may have crucial functions in development and neuronal function\(^12\), homozygous PrP knock-out mice generated by Büeler *et al*\(^1\) and Manson *et al*\(^10\) showed no overt developmental or behavioural abnormalities, but only subtle sleep defects\(^13\) and electrophysiological abnormalities suggesting that PrP may play a role in synaptic plasticity\(^14\), which could be reverted by re-introduction of a transgene encoding human PrP\(^15\). The *Prnp*\(^-/\)- mice generated by Sakaguchi *et al*\(^11\) instead presented with progressive ataxia from 70 weeks of age, caused by extensive loss of cerebellar Purkinje cells. Only recently, has it been discovered that this phenotype was not caused by the ablation of PrP function but by the activation of *doppel*, a gene 16 kb downstream of the *Prnp* locus\(^16\).

Re-introduction of mouse PrP transgenes in *Prnp* null mice restored their susceptibility to mouse prions in a dose-dependent fashion\(^17\), confirming an inverse relationship between the steady-state level of host PrP\(^C\) and the incubation time\(^18\). Instead, introducing a Syrian hamster *Prnp* gene into PrP null mice rendered these animals extremely susceptible to hamster prions, but made them almost resistant to mouse prions\(^19\).

The essential role of PrP\(^C\) itself in prion propagation was confirmed when *Prnp* knock-out mice were intracerebrally challenged with prions\(^19,20\). All three lines of these null mice not only lacked any clinical signs of scrapie disease but also showed unaffected brains, which did not harbour any infectivity or protease resistant PrP; thus, ablation of PrP\(^C\) abolished prion replication and propagation\(^10,11,19,21\).

At this point, it could be argued that PrP knockout mice were not affected because ablation of the normal host PrP removed the substrate for further conversion into PrP\(^Sc\). Hence, the role of PrP\(^Sc\)-mediated neurotoxicity had to be addressed differently. To achieve a sustained delivery of prions to the ‘prion-replication defective’ brains, we introduced neurografts expressing high PrP levels and inoculated them with prions\(^22\). These neurografts not only replicated and accumulated PrP\(^Sc\) and prion infectivity, but also delivered substantial amounts of prions to the host brain, notably without eliciting any clinical disease or neuropathological sign of spongiform encephalopathy. Hence, PrP\(^C\) is an indispensable substrate to elicit disease in the brain, and deposition of...
prions alone does not support neurodegeneration. These findings have kindled a series of further questions on the mechanisms of brain damage in TSEs. First, it is still obscure as to what eventually triggers neurodegeneration and what the targets in the CNS are. Second, how can low-level prion persistence in a subclinically affected subject be explained?

Mechanisms of cell death in prion disease

A large number of studies has been undertaken to analyse the role of PrP in neurodegeneration. Of particular interest are the function of PrPC in neuroprotection, and the mechanism of cell death induced by, or during, the conformational conversion from PrPC into PrPSc.

Despite much progress in the elucidation of the molecular pathways involved in the activation of cell death cascades, the mechanism of prion-induced cell death still remains obscure. Apoptotic cell death has been described in various cell culture systems and in vivo, and several hypotheses have been put forward to explain the neurotoxicity that leads to apoptosis, among them oxidative stress, microglia-mediated damage, and even the involvement of copper leading to increased levels of caspase 3, Fas activation, and up-regulation of the transcription factor c-jun.

Recently, the role of PrPC as a protein with anti-apoptotic function has been highlighted. It could be speculated that alteration of PrP function (e.g. by interaction with, or sequestration by, PrPSc) also affects a homoor heterodimerization of Bcl-2, or their expression, resulting in neuronal apoptosis. It has also been argued that binding and sequestration of ubiquitous members of the Bcl-2 family should also trigger cell death in non-neuronal cells. Since these show little or no alteration, further brain-specific factors, other than the Bcl-2 family members, may be more relevant. However, while loss of neurons can hardly be compensated, replacement of non-neuronal cells may happen unnoticed, particularly in the lymphoreticular compartment.

As will be discussed in detail below, overall loss of neurons may not be the most relevant event in prion disease. Patients may even be symptomatic through loss of neuronal connectivity and neurite degeneration, causing cell death only at a later stage.

Low level persistence

Mice harbouring only one wild-type allele of Prnp show partial resistance to scrapie infection, reflected by almost doubled incubation
times [Plate XI(A)]. While onset of symptoms and rate of disease progression apparently correlate with steady-state levels of host-PrP \text{C}, the disease severity did not as mice heterozygous for \textit{Prnp} showed a very benign course of disease. How can low-level persistence be explained in the framework of the protein-only hypothesis? Under typical experimental conditions, prions are inoculated into a suitable host, resulting in scrapie disease after an incubation time that is inversely correlated with the inoculated amount of infectivity. Typical applications are: (i) bioassays to determine the infectivity titre of a given inoculum by comparing it to a standard curve; or (ii) to investigate the influence of a specific treatment or a genetic defect (introduced by transgenesis) on the susceptibility to scrapie prions. However, all these experiments strongly rely on a reproducible response to a given dose of infectivity. This is the case, for example, for prion inocula derived from the Chandler strain (Rocky Mountain Laboratory, RML), transmitted into appropriate mouse lines. However, there are also reasons to believe that exposure to prions does not always result in clinical disease. As outlined above, \textit{Prnp}^{o/o} mice, which do not replicate prions, may nevertheless harbour low-level infectivity but never succumb to disease. These traces of prion infectivity were either ascribed to a low-level persistence of inoculum\textsuperscript{2} or were experimentally forced by an infected neurograft [Plate XI(B)]\textsuperscript{22}. Further examples, in which high-level prion titres accumulate without development of clinical signs of TSE, are immunodeficient mice, which have been peripherally challenged with prions\textsuperscript{9,33}.

In contrast to the above examples, in which a prion species barrier did not prevent replication, the exposure of humans to BSE involves crossing such a species barrier. Although a broad population in the UK has been exposed to BSE prions, only a relatively small fraction actually has developed the new variant of Creutzfeldt-Jakob disease (vCJD). This discrepancy could be explained by persistence of prions in lymphoreticular organs, which invariably takes place and is being used to diagnose preclinical vCJD\textsuperscript{34}; but the fact that there is also low-level, subclinical persistence of the BSE prions in the CNS cannot be excluded. This assumption is supported by one transmission study of BSE into mice, which then harboured infectivity that could be further passaged but did not always accumulate PrP\textsuperscript{Sc} in their brains\textsuperscript{35}. Similarly, mice inoculated with hamster prions accumulated prion infectivity in their brains and spleens, which was not ascribed to residual infectivity as transmission into \textit{Prnp} knock-out mice did not yield any infectivity\textsuperscript{36}. There is, however, an important parallel to the transmission of BSE to humans. In both settings, the incoming prions retained their strain specific attributes. The banding pattern of BSE and vCJD brains is indistinguishable in immunoblot analysis of brain extracts, indicating a
preservation of the BSE strain following transmission to humans\textsuperscript{[37]}, and infectivity found in wild-type mice that had received hamster prions retains the property of hamster, and not of mouse, prions as it elicited scrapie in hamsters but not in mice [Plate XI(C)]\textsuperscript{[36,38,39]. The replication of hamster prions in a mouse host (or of prions with a BSE pattern in human brains) is probably best explained\textsuperscript{[40] by an ‘over-rule mechanism’ in which the incoming prions strain passes its conformation over to the host PrP while converting it to PrP\textsuperscript{Sc}. This finding has important implications for farm-animal husbandry and the entry of animals into the food chain, if ingestion of BSE-contaminated food causes such animals to become subclinical carriers of BSE prions\textsuperscript{[41]. Detection of low-level infectivity in the absence of PrP\textsuperscript{Sc} is a considerable challenge for the development of future tests, but certainly not possible with the current detection methods for PrP\textsuperscript{Sc}.

**Clinical target areas: key to the understanding of scrapie neuropathogenesis?**

The importance of the selective vulnerability and injury of specific brain regions was first highlighted by Kimberlin and Walker\textsuperscript{[42,43] who proposed the clinical target areas in scrapie disease. Having studied neuro-invasion and spread within the CNS, they suggested that clinical signs evolve when neuronal damage, caused by whichever mechanism, exceeded a certain threshold\textsuperscript{[43]. After intracerebral inoculation, replication and accumulation of PrP\textsuperscript{Sc} and infectivity occurs at a nearly constant exponential rate until clinical disease develops. Following intraperitoneal inoculation, the thoracic spinal cord was the first region of the CNS to be colonised by prions, followed by a bi-directional spread towards lumbar and cervical spinal cord, respectively\textsuperscript{[42]. The disease interval to first detection of infectivity in the thoracic cord after intraperitoneal inoculation was shorter than the interval following intracerebral inoculation. Given the evidence for intra-axonal spread of prions within the CNS\textsuperscript{[22,44–46], the shorter disease interval following thoracic prion colonisation could be explained by a more speedy transport towards the clinical target areas, which may be located in brain stem nuclei. Kimberlin and Walker suggested that the differences may reflect the relative efficiency of the neural pathways by which infectivity spreads from different sites of entry in the brain to the postulated clinical target areas\textsuperscript{[42].

However, there are not many experimental approaches to strengthen this point. A possible approach to identify such target areas could be the injection of prions into these assumed target areas, or into a part of the CNS that is closely connected. One possible method of limiting the
spread of prions following injection would be stereotaxic implantation of metal or plastic spheres which had been coated with prion infectivity. The drawback of this approach would be the relatively limited accuracy and, whilst it would be possible to target a specific anatomical area, it would be limited to non-vital neuro-anatomical structures which are well circumscribed. Targeting a functional system, such as cholinergic or GABA-ergic neuronal circuits, would require a transgenic approach. Transgenic mice expressing PrP specifically in functional, physiologically relevant systems would be a feasible, though laborious, option, requiring dozens of transgenic mouse lines, each one expressing PrP under the control of a different promoter [Plate XII(A)]. This approach also suffers from variation in expression levels, due to integration effects and variability in copy number.

An elegant solution to this problem can be achieved by the use of inducible mouse mutants. One mouse line expressing the enzyme Cre recombinase under a cell- or region-specific promoter is crossed to another mouse line harbouring a gene flanked by LoxP sites, resulting in recombination and thus excision of the respective sequence in those cells which have expressed Cre recombinase [Plate XII(B–D)]. Several transgenic strategies are conceivable to address the role of clinical target areas:

1. The Cre-inducible expression of the PrP gene uses transgenic mice expressing a stop-cassette flanked by LoxP sites, followed by a PrP expression cassette. Cre expression results in activation of PrP only in those cells which undergo Cre-mediated recombination [Plate XII(D)]. The advantage over the classic transgenic approach is a better control of expression levels and a well-defined pattern of expression, but it has the drawback of being dependent on Cre-transgenic mouse lines. The ideal readout of such an experiment would be a significant difference in incubation times between different mouse lines expressing PrP in distinct neuronal circuits or anatomical regions.

2. The converse experiment aims at a reduction of clinical signs and hence the transgenic strategy has to be reversed: Either so-called conditional Prnp knock-out mice which carry a Prnp gene flanked by LoxP sites [Plate XII(B)] or, transgenic mice expressing a PrP expression construct flanked by LoxP sites [Plate XII(C)]47, crossed with suitable Cre-transgenic mice will selectively lose PrP in specific brain regions or cell types. Obviously, the readout would be a reduction of clinical signs and, perhaps, a milder disease progression.

3. While the above-mentioned approaches aim at a PrP reduction on a genomic level and are, therefore, suitable only for the proof-of-principle, the suppression on a post-transcriptional level would be more applicable to therapeutic strategies. Several recent studies have demonstrated that expression of antisense RNA effectively interferes with endogenous mRNA and suppresses transcription and translation of specific target genes.
Experimental verification of the functionality of this strategy would be the generation of transgenic mice, which express antisense RNA in neurons or in specific neuronal subsets [Plate XII(E)]. Inducible systems, such as the Tet-on/Tet-off system which uses a transactivator protein (tTA), composed of the repressor of the tetracycline operon (tetR) from *Escherichia coli* (Tn 10) and the activating domain of the herpes simplex virus protein 16 (HSV-VP16) \(^{49}\), or the Gal4/Lac system \(^{50}\) would allow for a controlled down-regulation of *Prnp* mRNA and might prove the feasibility and efficacy of therapeutic interventions aiming at a reduction of PrP\(^C\).

**Rationale for therapies aiming at a reduction of PrP expression**

Cellular PrP is undisputedly the substrate for PrP\(^\text{Sc}\)-mediated conformational conversion, and *Prnp* knock-out mice lacking this substrate are resistant to scrapie and do not propagate prions. Given the additional fact that ablation of PrP in the developed brain does not cause any negative adverse effect\(^{47,51}\), therapeutic approaches aiming at a reduction of PrP\(^C\) expression appear to be straightforward, although currently not easy to achieve\(^{40}\). However, whether removal of the substrate actually rescues the brain from a progressive clinical disease has yet to be clarified. Several experimental approaches are conceivable, all of them involving transgenic mice. Mice expressing PrP under control of a tTA-responsive promoter have been generated and have shown that down-regulation of PrP expression in the developed CNS is innocuous and that down-regulation of PrP expression prolongs the life span of infected animals, but data aiming at reduction of PrP expression are lacking.

Yet another strategy is the (irreversible) Cre-mediated inactivation of PrP. To achieve a temporally controlled reduction of PrP, an inducible Cre transgenic mouse line would be the method of choice for performing this experiment. The readout will be most useful for the understanding of scrapie pathogenesis. Of equal importance, it will indicate whether therapeutic approaches aiming at PrP suppression are a rational proposition.

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