Pathogenesis of prion diseases: current status and future outlook

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Abstract | The prion, a conformational variant of a host protein, is the infectious particle responsible for transmissible spongiform encephalopathy (TSE), a fatal neurodegenerative disease of humans and animals. The principal target of prion pathology is the brain, yet most TSEs also display prion replication at extra-cerebral locations, including secondary lymphoid organs and sites of chronic inflammation. Despite significant progress in our understanding of this infectious agent, many fundamental questions relating to the nature of the prion, including the mechanism of replication and the molecular events underlying brain damage, remain unanswered. Here we focus on the unresolved issues pertaining to prion pathogenesis, particularly on the role played by the immune system.

Iatrogenic transmission
The transmission of infectious agents as a consequence of a medical procedure.

Lymphoreticular system
The lymphoreticular system (LRS) is divided into primary and secondary lymphoid tissues. Primary lymphoid organs are anatomical sites where the cells of the LRS are generated, including the bone marrow and the thymus. Secondary lymphoid organs are sites where the LRS cells function. These sites include the spleen, the lymph nodes and mucosa associated lymphoid tissue. Tertiary lymphoid organs arise at sites of chronic inflammation.

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Transmissible spongiform encephalopathies (TSEs), or prion diseases, are inevitably fatal and there is no effective therapy available. The presence and impact of prion diseases on the human population, both past and present, is well established. Ritualistic anthropophagy of central nervous system (CNS)-derived tissues in New Guinea tribes resulted in the widespread incidence of the prion disease kuru, throughout this region. Indeed, kuru was a prime cause of death in Papua New Guinea until the middle of the twentieth century[1,2].

More recently, in the last quarter of the twentieth century, iatrogenic transmission of prion-contaminated gonadotropins to humans resulted in over 250 patients contracting Creutzfeldt-Jakob disease (CJD). Over the past 20 years more than 280,000 cattle have contracted bovine spongiform encephalopathy (BSE), an epidemic that provoked a worldwide food crisis with huge economic consequences. Over the same period, transmission of BSE to humans is believed to have caused over 150 cases of a new variant of CJD (vCJD)[3–6]. The possibility that millions of people could have come into contact with BSE-contaminated meat sparked a widespread public health scare that is still relevant today.

In addition, the occurrence of human-to-human prion transmission by blood transfusions alerted health authorities to the importance of vigorously controlling the origin and quality of blood donations[7]. Interestingly, some have suggested that prion diseases could have had a more sustained impact on human biology. It was proposed that variations in the human prion gene (*PRNP*) protecting against prion infection (for example, heterozygosity at codon 129) have disseminated more efficiently among human populations than non-protective polymorphisms, suggesting evolutionary selective pressure. Consequently prion diseases, now exceedingly rare, could have ravaged human populations in the distant past[8], although several publications have pointed out that these conclusions are open to interpretation[9–11].

Despite significant progress in our understanding of prion pathogenesis, there is still much to learn about prion tropism and transmission (BOX 1). Certainly, it is clear that the range of the disease extends beyond just brain pathology. For example, the disease-associated prion protein, PrP^{Sc}, was reported to be present in the spleen and muscle tissue of sporadic CJD (sCJD) patients[12] (FIG. 1), and prion infectivity was observed in the muscle of elk and deer suffering from chronic wasting disease (CWD)[13]. Moreover, as discussed below, chronic inflammation can extend the tropism of prion infectivity, and of PrP^{Sc}, to organs that were believed to be prion free (liver, pancreas, kidney, muscle and mammary gland)[14–17]. In addition, prion infectivity was also observed in the excreted urine of prion-infected, nephritic mice, a process termed prionuria[18]. These characteristics emphasize the complex distribution patterns of PrP^{Sc} and prion infectivity under varying pathophysiological conditions (for example, chronic inflammation) and in different hosts (for example, sheep, human, elk and deer). Indeed, involvement of the lymphoreticular system (LRS) seems to occur in most examples of TSEs, although the degree of involvement can vary depending on the prion strain (BOX 1), the host species and the polymorphisms of the *Prnp* gene[19–21]. Therefore, it is questionable whether only organs of the CNS and the LRS should be included in the current risk classifications of biologicals. It will be important to test

Box 1 | **A brief introduction to prions**

Prions are the agents of transmissible spongiform encephalopathy (TSE), with unconventional properties, such as resistance to high temperatures, high pressures, formaldehyde treatment or UV-irradiation. The term 'prion' does not have any structural implications other than that a protein is an essential component of the transmissible agent.

PrP^C. PrP^C is the naturally occurring cellular prion protein encoded by the *Prnp* gene. PrP^C is expressed on cells of the central nervous system and on cells of the immune system. In a given cell type PrP^C is necessary, but not sufficient for the replication of prions.

PrP^{Sc}. PrP^{Sc} is an abnormal isoform of the PrP^C protein and is found in the tissues of TSE patients. The isoform is partially resistant to digestion by proteinase K, is believed to be conformationally distinct from PrP^C and is considered to be the main component of the transmissible agent.

Prion strains. The concept of prion strains emerged from the finding that distinct versions of prion diseases, which differ at the symptomatic and biochemical level, can occur in the same mammalian species, even though the *PrP* gene is identical in these animals[154]. Strain-specific properties of prions could be encoded by a nucleic-acid genome, however, no evidence for this proposal has been forthcoming[155]. Alternatively, if the prion consists only of protein, PrP^{Sc} must exist in various distinct pathological conformations, each of which can impart its own conformation onto PrP^C, culminating in distinct pathologies. Strain phenotypes could be encoded in different conformations of PrP^{Sc} that have distinct stabilities against chaotropic salts and heat. These characteristics occur in the yeast Sup35 prion model[156–158].

altered prion tropism profiles in non-lymphoid organs and body fluids from farm animals and human patients with sCJD and vCJD.

The good news is that the number of BSE cases has dramatically decreased in almost all affected European countries over the past decade. However, sheep scrapie outbreaks and unexpected cases of BSE or vCJD continue to be reported even in countries and continents that were (more or less) prion free. To some extent, this could reflect the increased sensitivity of the latest generation of testing procedures but these data also underline our almost complete lack of knowledge about the transmission routes of prion diseases in humans and animals[22].

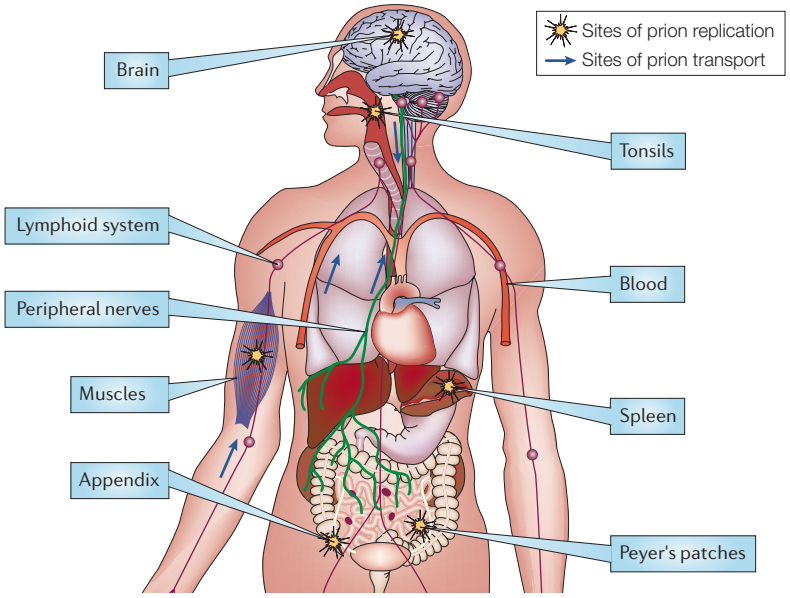

Figure 1 | **Human tissues and blood involved in propagation and transport of prions.** Orally ingested prions are intestinally absorbed and transported to the blood and lymphoid fluids. After a peripheral replication step in the spleen, appendix, tonsils or other lymphoid tissues, prions are transported to the brain primarily by peripheral nerves. Direct penetration into the brain across the blood–brain barrier is conceivable. Reproduced with permission from *Nature Reviews Microbiology* REF. 137 © (2004) Macmillan Publishers Ltd.

Important questions that remain unanswered include the nature of the infectious agent, the mechanisms of prion replication, the molecular properties underlying prion strains and the mechanistic basis of species barriers. Here, we focus on a specific area of investigation — extra-cerebral prion pathogenesis — and the following questions: why do prions accumulate in lymphoid organs and why do various states of immune deficiency prevent peripheral prion infection? How are prions transmitted between ruminants? What are cellular and molecular components that support human-to-human transmission by blood?

The cellular prion protein

PrP^C is a glycosyl phosphatidyl inositol (GPI)-linked glycoprotein that undergoes facultative *N*-linked glycosylation at two sites (FIG. 2a). Like other GPI-linked proteins, it is enriched in detergent-resistant membranes. The structure of mature PrP^C from mice, humans, Syrian hamsters and cattle shares common features: a long, flexible amino-terminal tail (residues 23–128), three α-helices, and a two-stranded anti-parallel β-sheet that flanks the first α-helix[23] (FIG. 2b). The second β-sheet and the third α-helix are connected by a large loop with interesting structural properties. This loop is extremely flexible in most species, but it is rigid in the prion protein of elk and deer[24] (FIG. 2c). It remains to be seen whether this structural peculiarity is connected to the propensity of deer and elk to develop chronic wasting disease. The carboxyl terminus of PrP^C is stabilized by a disulfide bond that links helices two and three[25] (FIG. 2a).

Even if the N-terminal portion of the molecule seems unstructured, it contains two defined, conserved regions. The first consists of a segment of five repeats of an octameric amino-acid sequence (octapeptide repeat region; OR)[23]. This region is proposed to be important in copper binding and could be involved in prion pathogenesis[26]. The second region, downstream relative to the first region, contains a highly hydrophobic and conserved profile, which was originally termed 'transmembrane region 1'. However, as it is unclear whether this domain really functions as a transmembrane region under physiological conditions, we propose to rename this region

the 'hydrophobic core' domain. It is preceded by a hydrophilic domain termed 'charge cluster' (FIG. 2a).

PrP^C is a highly conserved protein in mammals, and paralogues are present in turtles[27] and amphibians[28]. *Prnp* null alleles have not been observed in any mammalian species. The diverse and developmentally regulated[29] expression pattern of PrP^C in skeletal muscle, kidney, heart, secondary lymphoid organs and the CNS, indicates a conserved and broad function for the protein[6,30,31]. In the CNS, high PrP^C expression levels can be detected in synaptic membranes of neurons, and the protein is also expressed in astrocytes[32]. In the peripheral regions, PrP^C expression has been observed in lymphocytes and occurs at high levels in follicular dendritic cells (FDCs)[6,21].

What is the physiological function of PrP^C?

The *Prnp* gene was identified in 1986 (REF. 33) and *Prnp* knockout mice were first constructed in 1992 (REF. 34). Yet, despite these advances, an understanding of the function of PrP^C remains elusive. Many functions have been attributed to PrP^C, including immunoregulation, signal transduction, copper binding, synaptic transmission, induction of apoptosis or protection against apoptosis and many others[6]. PrP^C is expressed on long-term, re-populating hematopoietic stem cells[35] and the protein positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis[36]. However, postnatal depletion of PrP^C in neurons does not result in neurodegeneration[37]. Interestingly, neuronal

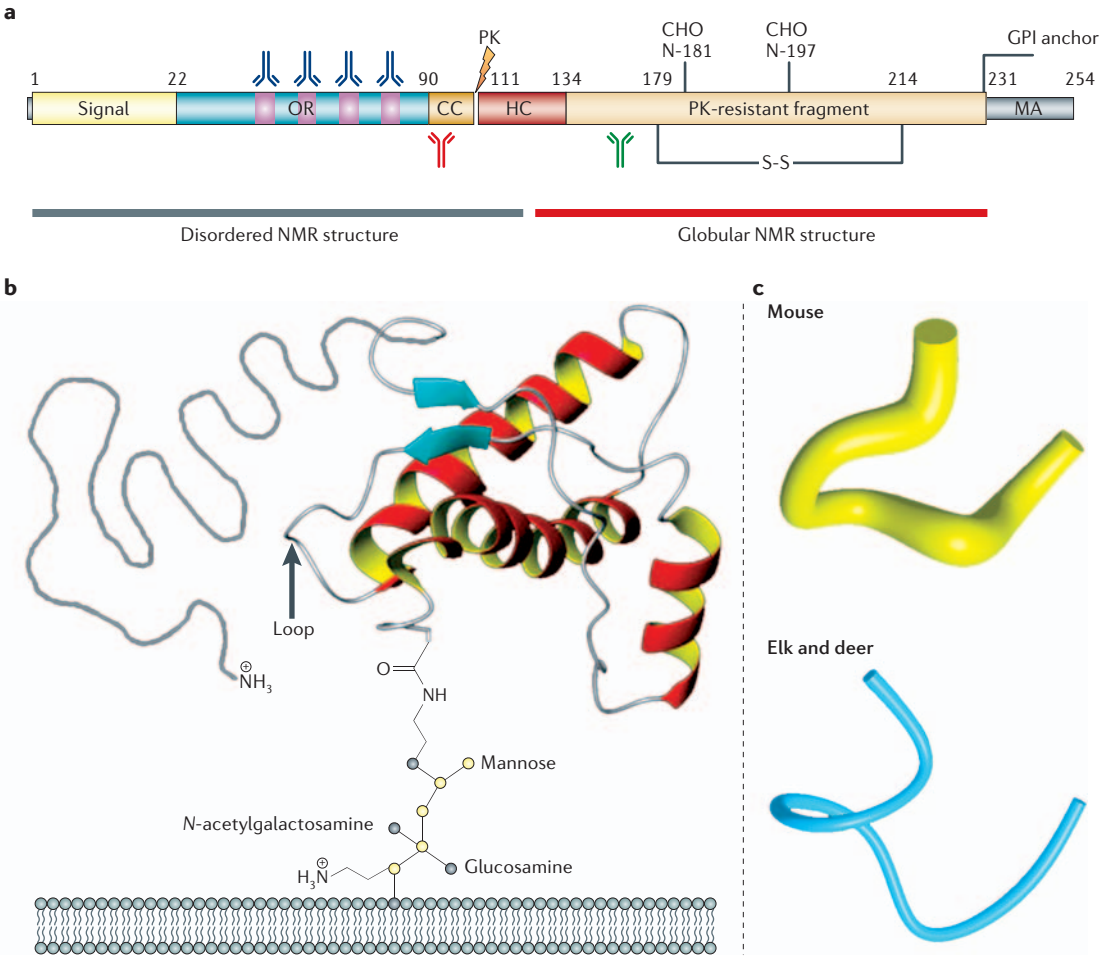

Figure 2 | **Structural features of the cellular prion protein. a** | An outline of the primary structure of the cellular prion protein including post-translational modifications. A secretory signal peptide resides at the extreme N terminus. The numbers describe the position of the respective amino acids. CC (orange) defines the charged cluster. HC (red) defines the 'hydrophobic core'. S-S indicates the single disulfide bridge. The proteinase K (PK) resistant core of PrP^{Sc} is depicted in gold and the approximate cutting site of PK within PrP^{Sc} is indicated by the lightening symbol. MA denotes the membrane anchor region. The epitopes recognized by the POM antibodies, some of which have extremely high affinities, are also indicated[138]. According to competition assays in solution and in surface plasmon resonance assays, POM2 (dark blue) binds to residues 58–64, 66–72, 74–80 and 82–88 (QPXXGG/SW); POM3 (red) to residues 95–100 (HNQWNK), and POM5 (green) to residues position 168–174. **b** | Tertiary structure of the cellular prion protein inserted into a lipid bilayer, as deduced from NMR spectroscopy, including the 'unstructured' N-terminal tail (grey) and the glycosyl phosphatidyl inositol (GPI) anchor. The loop connecting the second β-sheet and the third α-helix is indicated by the black arrow. OR, octarepeat region. **c** | The loop region is extremely flexible in most species (for example, mouse), but it is almost entirely rigid in the prion protein of elk and deer[24] as indicated by the average of the three dimensional space occupied during its oscillations. The figure shows amino acids 165 to 172 of the cellular prion protein of mouse, and elk and deer.

apoptosis was reported in the hippocampus and cerebellum following intracranial delivery of crosslinking anti-PrP antibodies[38]. However, regardless of its physiological function, the conversion of PrP^{C} to PrP^{Sc} culminates in neurodegeneration[6,39].

Prions and the immune system

Prion infections cause characteristic lesions in the CNS with spongiform vacuolation, accumulation of abnormal PrP^{Sc}, neuronal cell loss, microglial activation and proliferation of astrocytes. Neuronal cell loss, microglial activation and proliferation of astrocytes occur in many neurodegenerative diseases, but are particularly evident in prion diseases. In addition, prions colonize the lymphoid compartment of infected organisms. Following intracerebral (ic), oral or intraperitoneal (ip) inoculation, prion replication occurs in many sites of the LRS, including the spleen, lymph nodes, Peyer's patches (PPs) and tonsils[21,40–46] (FIG. 1). Following peripheral prion inoculation of C57BL/6 mice with the Rocky Mountain Laboratory (RML) prion strain, infectivity increases in the spleen and lymph nodes at approximately 30–50 days post inoculation (dpi), reaching a plateau after 6–9 weeks. However, brain infectivity is only detected after 4–5 months post-inoculation[44,47–50]. Even after ic inoculation with the RML strain, prion infectivity is found in spleens of wild-type mice at 4 dpi[48]. Indeed, splenic PrP^{Sc} is detectable 1 week post-inoculation and persists throughout the incubation period until the advent of terminal disease. Importantly, the spatiotemporal prion distribution patterns depend on the type and dose of the prion strain applied (for example, RML), and on the genetic background of the infected mice. Surprisingly, high prion titres in lymphoid organs are not accompanied by significant histopathological changes[51,52]. Indeed, there are no clear indications of immunotoxic effects of high prion infectivity titres in lymphoid organs[16], other than one report of abnormal germinal centre reactions[53].

Asplenia or splenectomy prior to, or shortly after, peripheral prion challenge prolongs the lifespan of scrapie-infected mice. Thymectomy or athymia has no effect[51], although the role of cervical thymi[54] , if any, remains to be assessed. In this model, peripheral prion pathogenesis becomes independent of the spleen after the infectious agent reaches the spinal cord[55]. However, splenic prion replication does not occur in all rodent TSE models: analysis of Syrian hamsters that had a splenectomy and which were subsequently infected (ip) with the 236K strain showed that neuroinvasion did occur without substantial prion replication in the LRS[56]. Additionally, splenectomy had no influence on incubation times after infection with a mouse-adapted CJD prion strain[57,58].

Separation of splenic pulp from the stroma revealed that approximately tenfold more infectivity was present in the stroma compared with the pulp fraction. Moreover, infectivity in the stromal fraction directly correlated with both whole spleen weight and the weight of the stroma. It was concluded that the stromal compartment is the site of replication of the scrapie agent[59], and that the cells involved in scrapie replication were not mitotically active[42]. Accordingly, sublethal irradiation, which preferentially targets mitotically active cells, failed to alter the incubation period of the disease[60].

PrP^{C} and peripheral prion pathogenesis

If prions multiply by imparting their conformation on to the PrP^{C} protein, organisms devoid of PrP^{C} should be resistant to scrapie. Indeed, lack of the *Prnp* gene confers absolute resistance against cerebral and peripheral scrapie infection[48], and the presence of PrP^{C} is essential for neuronal damage in infected individuals[61].

PrP^{C} itself is involved in transporting prion infectivity from peripheral sites to the CNS. Adoptive transfer of wild-type bone marrow into $Prnp^{o/o}$ mice reconstitutes the ability of the spleen to accumulate high titres of prion infectivity up to 300 dpi (REFS 62,63). However, this process was insufficient to restore prion neuroinvasion. Therefore, hematopoietic cells (for example, B and T cells, macrophages and dendritic cells) facilitate the transport of prions from the peripheral entry site to secondary lymphoid organs, where prions can accumulate and/or replicate. However, the primary cellular reservoir for prion neuroinvasion seems to be non-hematopoietic, because this phenotype cannot be restored by bone marrow reconstitution[62–64].

In conclusion, following peripheral exposure — whether by ingestion or by peripheral infection — prion pathogenesis is a dynamic process consisting of spatially and temporally distinct phases. After initial infection and peripheral prion replication, prions migrate from the peripheral regions of the host to the CNS. Finally, fatal progressive neurodegeneration, spongiosis and gliosis develop in the infected host.

Cytokines, chemokines and prion neuroinvasion

The precise definition of the cells and molecules enabling peripheral prion replication and neuroinvasion has increased our understanding of prion pathogenesis. B lymphocytes are crucial for peripheral prion spread and neuroinvasion[64]. However, PrP^{C} expression on B lymphocytes is not compulsory for prion neuroinvasion[63,65,66]. Prion neuroinvasion, combined with the fact that a stromal compartment is the essential mediator of prion invasion[59,62], indicates that B lymphocytes are unlikely to be a major 'replicative compartment' for prions in mice. Instead, the apparent requirement for B lymphocytes in peripheral pathogenesis is more likely to be derived — at least in part — from indirect effects, including the provision of chemokines or cytokines to cells that efficiently replicate prions in peripheral regions of the host body. Any potential profiteer from these B-lymphocyte-derived signals would be localized in close proximity to the B lymphocytes, be of stromal origin[59] and should also display PrP^{C} on its cell surface. FDCs fulfill each of these criteria. Under naïve conditions, FDC networks are only present in small amounts in B-lymphocyte follicles. However, upon activation, large FDC networks are formed, with long protrusions that interact with lymphocytes[67,68].

Peripheral prion inoculation
This defines any administration of the prion agent other than into the central nervous system, including intraperitoneal (ip), intravenous (iv), oral or intraocular (io) administration.

Splenic stroma
The splenic stroma defines those cells in the spleen, which are of non-hematopoietic origin and are resistant to γ-irradiation.

Splenic pulp
The splenic pulp can be subdivided into the red and the white splenic pulp. The splenic red pulp fills the sinuses of the spleen and its composition includes macrophages and red blood cells. The white splenic pulp is a parenchymatous tissue of the spleen consisting of compact masses of lymphatic cells and contains the germinal centres.

FDCs support the formation and maintenance of the lymphoid microarchitecture by expressing homeostatic chemokines (for example, CXCL13), and have a role in antigen trapping and capturing of immune complexes by Fcγ receptors. FDCs bind opsonized antigens through the CD21/CD35 complement receptors[67]. So far, no exclusively FDC-specific genes have been identified, and the nature of the FDC-M1 antigen remains elusive[16,69]. Nevertheless, various protocols to enrich for, or to deplete, FDCs have provided useful knowledge about genes expressed by FDC-M1 positive clusters[70,71].

Besides initiating and controlling immune responses in the splenic white pulp and in B-lymphocyte zones of PPs and lymph nodes, B lymphocytes also express lymphotoxin (LT), tumour necrosis factor (TNF), and other factors that contribute to the maturation and maintenance of FDCs[72,73]. LTα and LTβ are pro-inflammatory cytokines originally recognized for their cytotoxic effects on normal and transformed cells *in vitro* (and therefore have also been referred to as cytotoxins)[74,75]. LTs belong to a family of structurally related cytokines — the TNF superfamily[72,76] — and are expressed primarily by B lymphocytes and activated T lymphocytes. Ectopic expression of LT in the liver, kidney or pancreas leads to the generation of follicular structures, resembling tertiary follicles[14,77,78], which are very similar to activated lymphoid follicles found in the spleen or lymph nodes. Therefore, LT molecules contribute to both the generation and maintenance of FDCs under physiological and immunopathological conditions.

Lymphoid microarchitecture and prions

FDCs accumulate PrP^{Sc} following scrapie infection[79], and prion replication in the spleen was reported to depend on PrP^{C}-expressing FDCs — at least in the ME7 prion strain[80]. Conversely, mice devoid of FDCs and lacking an organized microarchitecture (for example, $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $LT\beta R^{-/-}$ mice) show reduced or impaired prion replication in lymphoid tissues following peripheral exposure[81]. Interestingly, prion inoculation experiments with mice lacking FDCs revealed that other cell types were capable of replicating prions, at least in the lymph nodes[81]. Therefore, poorly defined immune cells or possibly stromal precursor cells (for example, for FDCs) might also be capable of replicating the infectious agent[81,82].

Inhibiting the LTβR pathway in mice and nonhuman primates (by treatment with LTβR-immunoglobulin fusion protein (LTβR-Ig)) results in the disappearance or de-differentiation of mature, functional FDCs[83,84]. In addition, treatment with LTβR-Ig was found to impair peripheral prion pathogenesis[85,86]. Treatment of mice with LTβR-Ig for one week prior to exposure to a relatively low dose (ip) of prions, followed by further treatment for two weeks post-inoculation, resulted in the complete protection of the mice from disease[16].

FDCs, the complement system and prion uptake

As FDCs interact with opsonized antigens through the CD21/CD35 complement receptors, a pertinent question is whether there is a role for the complement cascade in the pathology of prion diseases? Indeed, mice that lack various complement factors, including C1q[87], or have been depleted of the C3 complement component[88], have enhanced resistance to peripheral prion inoculation. Human studies also point to a possible role for members of the classical complement cascade in the biology of prion pathogenesis[89,90]; however, their precise role in prion disease is unknown. In future experiments, it will be important to ascertain whether FDC-associated complement receptors have a role in promoting the efficiency and transport of prion infectivity in extra-neural compartments, and to explore how components of the classical complement cascade respond to peripheral prion infection.

How do prions migrate into the nervous system?

The innervation pattern of lymphoid organs is primarily sympathetic[91], and many studies suggest that the autonomic nervous system might be responsible for the transport of the prion agent from lymphoid organs to the CNS[59,92,93] (BOX 2). Unsurprisingly, the sympathetic nervous system is affected in vCJD patients[94]. Sympathectomy delays the onset of scrapie following ip inoculation of the infectious agent, whereas sympathetic hyperinnervation enhances splenic prion replication and neuroinvasion. These findings suggest that innervation of secondary lymphoid organs is a rate-limiting step in the pathway to neuroinvasion[95]. The detection of PrP^{Sc} in the spleens of sCJD patients[12] indicates that the interface between cells of the immune and peripheral nervous systems might also be of relevance in sporadic prion diseases.

Box 2 | **The nervous system**

The autonomic nervous system (ANS) regulates individual organ function and homeostasis and, for the most part, is not subject to voluntary control. It is divided into the parasympathetic, sympathetic and enteric systems on the basis of anatomical and functional differences. Each of these systems consists of myelinated pre-ganglionic fibres which make synaptic connections with unmyelinated post-ganglionic fibres, and it is these which then innervate the effector organ.

Sympathetic nerves originate inside the vertebral column, toward the middle of the spinal cord in the intermediolateral cell column (or lateral horn), beginning at the first thoracic segment of the spinal cord and extending into the second or third lumbar segments. Because its cells begin in the thoracic and lumbar regions of the spinal cord, the sympathetic nervous system is said to have a thoracolumbar outflow. Axons of these nerves leave the spinal cord in the ventral branches (rami) of the spinal nerves, and then separate out as 'white rami' which connect to two chain ganglia extending alongside the vertebral column on the left and right. These elongated ganglia are also known as paravertebral ganglia or sympathetic trunks. In these hubs, connections (synapses) are made which then distribute and direct the nerves to major organs (for example, spleen), glands and other parts of the body.

Homeostatic chemokine
A subset of the chemokine family that are constitutively expressed in pre-formed lymphoid tissues and which promote and maintain the organization of this tissue.

FDC-M1 positive cluster
A dense network of cells found in germinal centres, immunoreactive for the FDC-M1 antibody and the CD21/35 receptor. Tingible body macrophages also stain positive for FDC-M1 but are morphologically distinct.

Lymphotoxin
(LT). LTα and LTβ are proinflammatory cytokines that belong to the tumour necrosis factor (TNF) superfamily. They are mainly expressed by B- and T lymphocytes, and natural killer cells. LTs exist as membrane-bound heterotrimers ($LT\alpha_1\beta_2$ or $LT\alpha_2\beta_1$) or as secreted homotrimers ($LT\alpha_3$). LTs bind TNFR1 or LTβR inducing a signalling cascade that is important for the maturation and maintenance of follicular dendritic cells.

Ectopic expression
This defines the expression of a gene in an abnormal site in an organism. This phenomenon can be induced by disease or by a pathogen, but can be also induced artificially by expressing a transgene with a tissue or cell-specific promoter.

LTβR pathway
Following interaction with lymphotoxin ligands, LTβR can activate an 'alternative' pathway for NFκB, inducing the expression of genes such as homeostatic chemokines and tumour necrosis superfamily members, which is important for the maintenance and maturation of follicular dendritic cells.

Extra-neural compartment
This includes organs and cells that do not belong to the central or peripheral nervous system.

Innervation pattern
This describes the type (qualitative and quantitative) of innervation present in a peripheral organ that does not belong to the central nervous system.

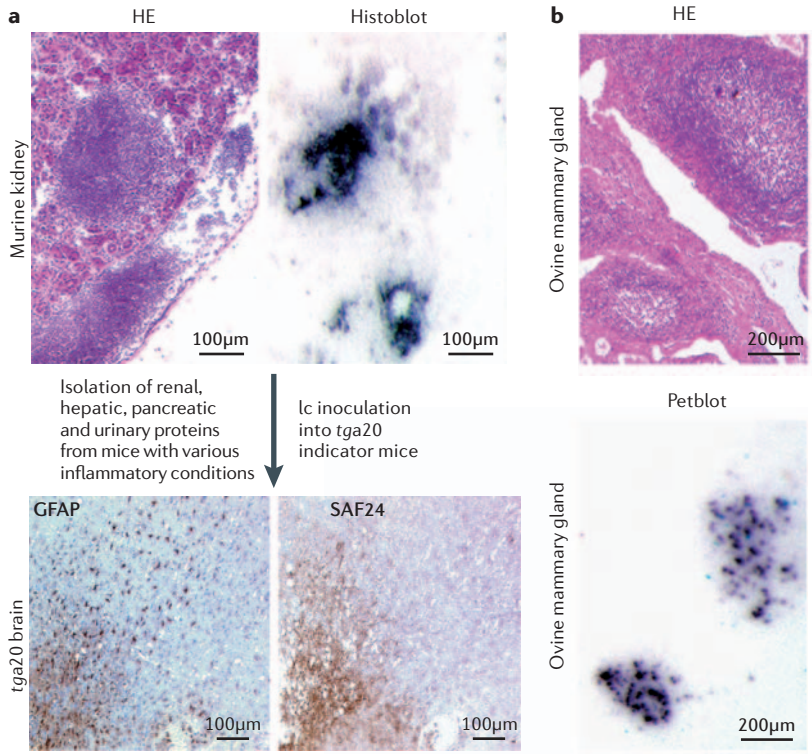

Figure 3 | **PrP^{Sc} and prion infectivity in chronically inflamed murine or ovine tissue.** **a** | HE stained section of renal tissue derived from a prion-infected (RIPLTα) mouse. Inflammatory foci that overlap with PrP^{Sc} deposition can be detected by histoblot analysis of a renal cryosection blotted on a nitrocellulose membrane followed by proteinase K (PK) treatment. Indicator mice (*tga20* mice) can be inoculated with renal, hepatic and pancreatic homogenates, and urinary proteins, derived from various transgenic or spontaneous mouse models for pancreatitis, hepatitis or nephritis (lower panel). Prion disease present in *tga*20 indicator mice can be visualized by immunohistochemical analysis of consecutive brain sections. This analysis indicates PrP deposits (SAF84 staining) and strong astrogliosis (GFAP staining). **b** | HE staining of a mammary gland derived from sheep infected with scrapie and presenting with mastitis. As above, PrP^{Sc} deposition coincides with tertiary follicles , as confirmed by petblot analysis of a paraffin section blotted on a nitrocellulose membrane, followed by PK treatment (lower panel). To determine whether milk, urinary proteins, and possibly other secretions from scrapie-sick sheep contain prion infectivity, transgenic indicator mice, other rodents such as bank voles[159] or *in vitro* scrapie cell assays[150] can be inoculated with this material. HE, hematoxylin and eosin. HE-stained section in part **a** reproduced with permission from REF. 14 ©(2005) American Association for the Advancement of Science (AAAS). GFAP and SAF24 sections in part **a** reproduced with permission from REF. 18 © (2005) AAAS. HE-stained section in part **b** reproduced with permission from *Nature Medicine* REF. 15 © (2004) Macmillan Publishers Ltd.

The distance between FDCs and splenic nerves impacts on the rate of neuroinvasion[96]. FDC positioning was manipulated by ablation of the CXCR5 chemokine receptor, directing lymphocytes towards specific microcompartments[97]. As such, the distance between germinal centre-associated FDCs and nerve endings was reduced[96,97]. This process resulted in an increased rate of prion entry into the CNS in CXCR5$^{-/-}$ mice, probably owing to the repositioning of FDCs in juxtaposition with highly innervated, splenic arterioles. It remains to be determined whether the increased rate of neuroinvasion results from a passive diffusion of prions (for example, released FDC exosomes[98, 99]), or whether mobile cells, such as dendritic cells (DCs) or B lymphocytes located in the germinal centre, are involved in an active-transport process. Other mobile elements, including budding viruses, could also function as vehicles for infectivity[100]. Alternatively, prion infectivity could directly invade the brain by the blood–brain barrier[101]. The cellular and molecular preconditions for such a process remain elusive.

Inflammation: a license to replicate?

As lymphoid infectivity occurs in most examples of prion disease, and proinflammatory cytokines and immune cells are involved in lymphoid prion replication[79–81,85,96,102], we assessed whether chronic inflammatory conditions in non-lymphoid organs could affect the dynamics of prion distribution. Indeed, inclusion body myositis, an inflammatory disease of muscle, was reported to lead to the presence of large PrP^{Sc} deposits in muscle[17]. Many chronic inflammatory conditions, some of which are very common and include rheumatoid arthritis, type-I diabetes, Crohn's disease, Hashimoto's disease and chronic obstructive pulmonary disease, result in organized inflammatory foci of B lymphocytes, FDCs, DCs, macrophages and other immune cells associated with germinal centres[103–106]. In addition, extranodal metastases of Hodgkin's disease and non-Hodgkin's lymphomas might contain neoplastic follicles containing FDCs[107,108]. Furthermore, the meninges can also develop ectopic, lymphoid follicles under conditions of chronic inflammation[109]. Most importantly, tertiary follicles can be induced, and are surprisingly prevalent, in non-lymphoid organs by naturally occurring infections in ruminants[106,110].

To investigate whether inflammatory diseases influence prion pathogenesis, mice with five different inflammatory diseases of the kidney, pancreas or liver were inoculated with the RML prion strain[14]. In all cases and in all organs tested, chronic lymphocytic inflammation resulted in prion accumulation in otherwise prion-free organs. The presence of inflammatory foci consistently correlated with an upregulation of LT and the ectopic induction of PrP^{C}-expressing FDC cells. Inflamed organs of mice lacking LTα or LTβR did not accumulate either PrP^{Sc} or infectivity following prion inoculation. Scrapie infection of mice suffering from nephritis, hepatitis or pancreatitis also induces unexpected prion deposits at the sites of inflammation[14] (FIG. 3).

These data have raised concerns that analogous phenomena might occur in farm animals, since these are commonly in contact with inflammogenic pathogens. Indeed, PrP^{Sc} has been observed in the inflamed mammary glands of sheep with mastitis and which are also infected with scrapie[15] (FIG. 3). These observations indicate that inflammatory conditions induce accumulation and replication of prions in organs previously considered to be free from prion infection. If confirmed, these findings could have an impact on the risk assessment of dairy foodstuffs (for example, milk) and could lead to a readjustment of current rules.

In addition, it was proposed that inflammatory conditions could result in the shedding of the prion agent by excretory organs, including the kidney. To investigate this hypothesis, various transgenic and spontaneous mouse

models of nephritis were analysed to ascertain whether prions could be excreted in urine[18]. Prion infectivity was observed in the urine of mice with both subclinical and terminal scrapie, and in mice with inflamed kidneys[18].

The factors that allow the spread of prion infectivity between hosts have been a source of contention for over 100 years. It is possible that the horizontal spread of prions between hosts is mediated by secreted body fluids (for example, urine and milk) that are derived from potentially infectious secretory organs (for example, kidney and mammary glands). It was also proposed that the placenta of infected ewes could provide a source of prion infectivity for horizontal transmission to new hosts[111]; however, so far, there is little data to support this hypothesis.

Public health considerations mandate that we should increase our understanding of the altered prion tropism observed in ruminants (including sheep, cattle, goat, elk and deer) and the underlying mechanisms, especially those related to inflammation. Future experiments should include an analysis of the effect of other common chronic inflammatory disorders (for example, granulomatous diseases) in prion-infected rodents and farm animals. Moreover, it remains unclear whether prion infectivity is actively or passively transported into tertiary follicles. As LT is upregulated in almost all states of inflammation and LTα$^{-/-}$ or LTβR$^{-/-}$ mice do not support prion replication in organs with lymphocytic inflammation, it is reasonable to suggest that LT has an important role in the induction of ectopic prion replication[112]. Indeed, it is plausible that LT itself imparts prion replication competence to various cell types (for example, stromal or mesenchymal cells). Future experiments will test the hypothesis that LT, induced by various exogenous stimuli (viral, bacterial or parasitic infection), promotes a cellular microenvironment capable of prion replication (FIG. 4). It is possible that different physiological states, such as the number of PPs[113] or the presence of prion infectivity in blood[114], alter the molecular and cellular preconditions required for ectopic prion accumulation and replication.

Portals of entry

Following oral infection, an early increase in prion infectivity is observed in the distal ileum. This occurs in several species, but has been most extensively investigated in sheep and deer[19,115,116]. Western blot analyses and bioassays have shown that PPs accumulate PrP^{Sc} and contain high titres of prion infectivity. Similarly, after inoculation with mouse-adapted scrapie prions (RML strain), mice experience a surge in intestinal prion infectivity as early as a few days post-inoculation[113,117]. However, early accumulation of PrP^{Sc} was also found in the enteric nervous system and gut-associated lymphoid tissue (GALT) of hamsters that were orally infected with scrapie[46], suggesting an alternative route of infection in this model. Immune cells are crucially involved in the process of neuroinvasion following oral administration: mature FDCs, located in PPs, could be crucial for the transmission of scrapie from the gastrointestinal tract[112,113,117]. The cellular basis for prion transmigration from the gut through the GALT into the lymphoid system is still poorly understood. Membranous epithelial cells (M cells) are believed to be the key sites of antigen sampling for the mucosal-associated lymphoid system, and function as major ports of entry for enteric pathogens from the gut by transepithelial transport[118,119]. Interestingly, maturation of M cells is dependent on signals transmitted by intraepithelial B lymphocytes. Efficient *in vitro* systems have been developed, in which epithelial cells can be instructed to undergo differentiation to cells that resemble M cells, as judged by morphological and functional-physiological criteria[120]. This led to the proposal that M cells could be a site of prion entry — a hypothesis that has been substantiated in co-culture models[121]. DCs, being mobile, could function as a bridge between the gut lumen and the lymphoid TSE replicative machinery[122]. Indeed, splenic CD11c^{+} cells, isolated from scrapie-infected donors and injected intravenously into RAG1$^{-/-}$ mice, induced scrapie without the accumulation of prions in the spleen[123]. As such, it is tempting to speculate that DCs could transport prions from their sites of replication to peripheral nerves in lymphoid organs, thereby enabling the process of neuroinvasion.

Prion transmission through blood

Prion infectivity can reside in the blood of sheep and humans. Moreover, prions were reported to be transmitted by animal blood transfusion prior to the onset of clinical signs[114,124]. This potential for inadvertent transmission of the vCJD agent to humans by blood transfusion was often regarded as a 'hypothetical' risk. However, we now know that the risk is not hypothetical, and three cases of transfusion-related transmission of vCJD have been reported[11,125,126], with the likelihood of additional cases in the future[125]. Although the number of affected individuals is small, it represents a high proportion of the maximum number of possible cases, based on the number of people that are known to have received prion-contaminated blood. Consequently, the possible contamination of blood products with prions will be a significant problem for transfusion medicine for the foreseeable future. Screening for contaminated blood products will become important when the appropriate methodologies are available. In addition, focusing research on the following questions will be crucial to tackling this problem effectively: first, which blood-borne cells have prion infectivity?; second, which plasma proteins associate with prions[127]?; third, are there strain- and species-specific differences between sheep and humans in terms of the distribution and stability of blood-borne prions?; fourth, when — following initial infection — does prion infectivity arise in blood?; and finally, do generic or specific inflammatory states increase the likelihood of blood-borne prion infectivity?

Prion degradation

Although prions are resistant to high temperatures, high pressure, formaldehyde treatment and UV-irradiation, it is not possible to recover prions from *Prnp*$^{o/o}$ mice following peripheral infection with relatively high doses of the RML strain. Therefore, there must be mechanisms that mediate the efficient removal of prion infectivity. Understanding and enhancing such mechanisms, perhaps through activation of putative prion-degrading

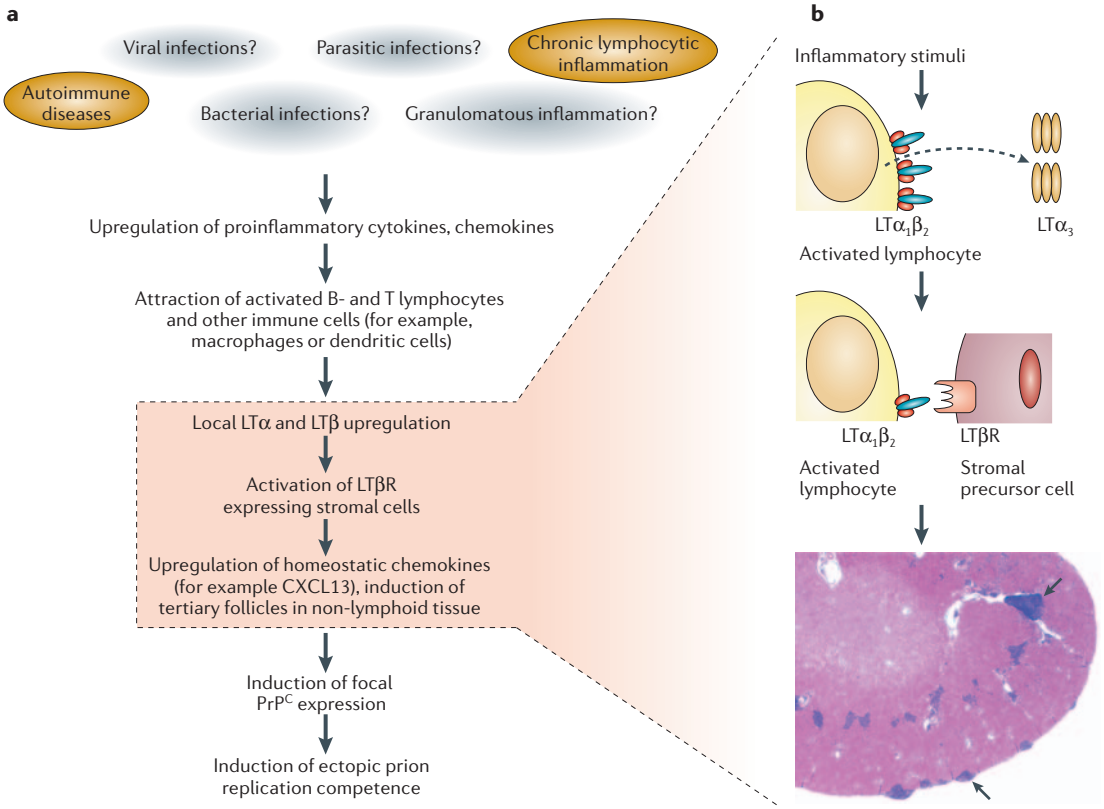

Figure 4 | **Induction of tertiary follicles and prion replication competence in non-lymphoid tissue. a** | A hypothetical hierarchical cascade resulting in the generation of tertiary follicles in non-lymphoid tissue, with the possible induction of ectopic prion replication competence. Autoimmune diseases and chronic lymphocytic inflammations have been demonstrated to induce prion replication competence in non-lymphoid tissue, whereas other conditions have not been investigated in this regard yet (indicated by the question marks). **b** | A model of the events that induce the generation of tertiary follicles and prion replication competence in the context of inflammatory conditions. Activation of B- and T lymphocytes induced by inflammatory stimuli upregulates lymphotoxin (LT) expression on lymphocytes (for example, on B lymphocytes). LT secreted by ($LT\alpha_3$), or expressed on the extracellular membrane ($LT\alpha_1\beta_2$) of B lymphocytes, binds to LTβR (or TNFR1) of stromal precursor cells, inducing the upregulation of adhesion molecules, chemokines and/or cytokines. The effect of these events is the induction of tertiary lymphoid follicles and prion replication competence. A HE section of an inflamed kidney is shown (lower panel). Small arrows indicate follicular inflammation. Section image reproduced with permission from REF. 18. © (2005) American Association for the Advancement of Science.

cells, could be a possible strategy to treat prion diseases. So far, however, the most promising results relating to treatment strategies focus on immunosuppression, including conditional de-differentiation of FDCs with LTβR-Fc, de-complementation by cobra-venom factor, or the suppression of germinal centres and disruption of lymphoid microarchitecture.

We and others have asked the question of whether pattern-recognition receptors, such as the members of the Toll-like receptor (TLR) family, might be involved in the recognition and subsequent degradation of prions. The hypothesis was enticing because the ordered aggregate state of PrP^{Sc} could theoretically render it recognizable as a pathogen-associated molecular pattern. However, the kinetics of prion pathogenesis in $MyD88^{-/-}$ mice inoculated with prions (by the ip route) are identical to the kinetics observed in the wild-type control mice[128], suggesting that signalling by TLR1, 2, 6, and 9 (mediated by the adapter protein MyD88 (REF. 129)) are probably not involved in prion recognition and signalling. However, targeting TLR9 therapeutically might have other beneficial effects. TLR9 recognizes DNA sequences that are overrepresented in bacterial DNA. For example, unmethylated cytosine phosphate guanosine (CpG) motifs present in bacterial DNA can stimulate mouse and human immune responses through TLR9 (REF. 130). Repetitive CpG motifs in synthetic oligodeoxynucleotides (CpG–ODN) simulate bacterial unmethylated nucleic-acid sequences, and thereby stimulate the innate immune system through TLR9 expressed on various immune cells, including monocytes, macrophages and dendritic cells. CpG–ODN treatment has been discussed as a possible therapy to delay prion disease, primarily based on promising results in mouse scrapie[131]. The delay in the development of prion disease in this model could be due to the destruction of the primary site of peripheral prion amplification — the lymphoid follicles[132]. Alternatively, the massive expansion of macrophage and dendritic cells that is evident following repetitive CpG–ODN treatment might lead

to enhanced PrP^{Sc} degradation or prion sequestration. Macrophages might conceivably function as prion transporters when exposed to high prion titres; however, they might also inhibit prion infectivity when confronted with manageable prion titres[133]. However, despite these promising results, CpG–ODNs are not likely to be a viable anti-prion therapy owing to the severe toxic side effects associated with repeated administration. An interesting approach might consist of phagocytosis activation and prion degradation without the reported adverse side effects associated with CpG–ODNs[19].

Surrogate markers and new tools

PrP^{Sc} is not always easily detectable in prion diseases[134–137]. Therefore, the development of highly sensitive assays for biochemical detection of PrP^{Sc} in tissues and body fluids is a top priority. One route to achieve this goal is to develop high-affinity immunoreagents that recognize PrP^{Sc}. Examples include the 'POM' series of antibodies that recognize various well-defined conformational epitopes in the structured C-terminal region of PrP^{C}, and linear epitopes in the unstructured N-terminal region (FIG. 2)[138]. Because of the particular nature of the epitopes to which they are directed, some of these antibodies have affinities for the prion protein in the femtomolar range (Magdalini Polymenidou, personal communication). Protein misfolding cyclic amplification (PMCA) is also a promising method for the sensitive detection of the pathological prion protein[139,140]. This method relies on the principle of disrupting large PrP^{Sc} aggregates by sonication to generate multiple smaller units. PMCA was recently shown to increase sensitivity 6,600-fold over standard detection methods[141].

Amplifiable PrP^{Sc} was detected in the blood of scrapie-infected hamsters by PMCA during most of the pre-symptomatic phase of disease[142]. Several research efforts have also been directed at identifying proteins that are differentially expressed in the tissues of prion-infected animals compared with disease-free control animals[143–145]. Ideally, these surrogate markers should be detectable at preclinical stages of disease and be differentially expressed in easily accessible body fluids such as blood or urine. So far, only one extra-neural gene — the erythroid differentiation related factor — has been identified that is differentially expressed during prion infection of experimentally infected mice, cattle with BSE and sheep with clinical scrapie[146]. The identification of additional surrogate markers would certainly be useful, particularly if they are detectable in body fluids. Although surrogate markers such as S-100, neuron-specific enolase, and 14-3-3 protein have been suggested as potential biomarkers of prion disease in body fluids, including cerebral-spinal fluid[147,148], they are not specifically predictive of human prion disease.

The gold standard of prion diagnostics is the ability to detect prion infectivity itself. Until recently, the animal bioassay was the only method available to screen for prion infectivity, for example, by using transgenic mice overexpressing PrP^{C} (*tga*20) (REF. 149). However, this bioassay is imprecise, takes 6–7 months to complete and is very costly. More recently, the development of highly susceptible, cloned neural cell lines (PK1 N2a cell line) have provided an assay that improves the precision, cost and time required to do prion detection bioassays, and might lend itself to high-throughput automation[150].

Such assays have the potential to advance methodologies aimed at the diagnostic assessment of whether the prion agent is present. It should be noted, however, that these cell lines are currently reported to be permissive to only murine prions. Future experiments will hopefully provide the means by which the full range of prion strains and species can be assayed. Perhaps the establishment of a human immune system in the mouse could be an efficient tool to test the potential human pathogenicity of various prion strains *in vivo*[151–152].

The relationship between infectivity, PrP^{C}-converting activity and the size of various PrP^{Sc}-containing aggregates has been systematically investigated. In this analysis, PrP^{Sc} aggregates were partially fragmented, fractionated by size and assessed for infectivity and converting activity[153]. The analysis revealed that 17–27 nm-sized (300–600 kDa) particles had the highest infectivity and converting activities, whereas these activities were substantially lower in large fibrils and virtually absent in oligomers containing ≤ 5 PrP^{Sc} molecules. Therefore, non-fibrillary PrP^{Sc}-containing particles with masses equivalent to 14–28 molecules are the most efficient initiators of prion infection.

The future of prion science

Considerable knowledge on the biology of prions has been amassed over the past decade, yet many questions remain unanswered, including some relating to the most basic aspects of prion biology. What is the precise physical nature of the prion? What is the biochemical basis of prion strains? What factors determine the species barriers in prion infections? What are the host susceptibility factors that promote prion infection? And, finally, what are the molecular mechanisms that will underpin successful sensitive diagnostics[137] and efficacious therapies? The tools and experimental models available now should make it possible to answer many of these questions. The development of new technologies, and the input of fresh ideas, has opened up new perspectives on our understanding of the mechanisms of central and peripheral prion pathogenesis, some of which could be applicable to other neurodegenerative diseases.

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Competing interests statement

The authors declare no competing financial interests.

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