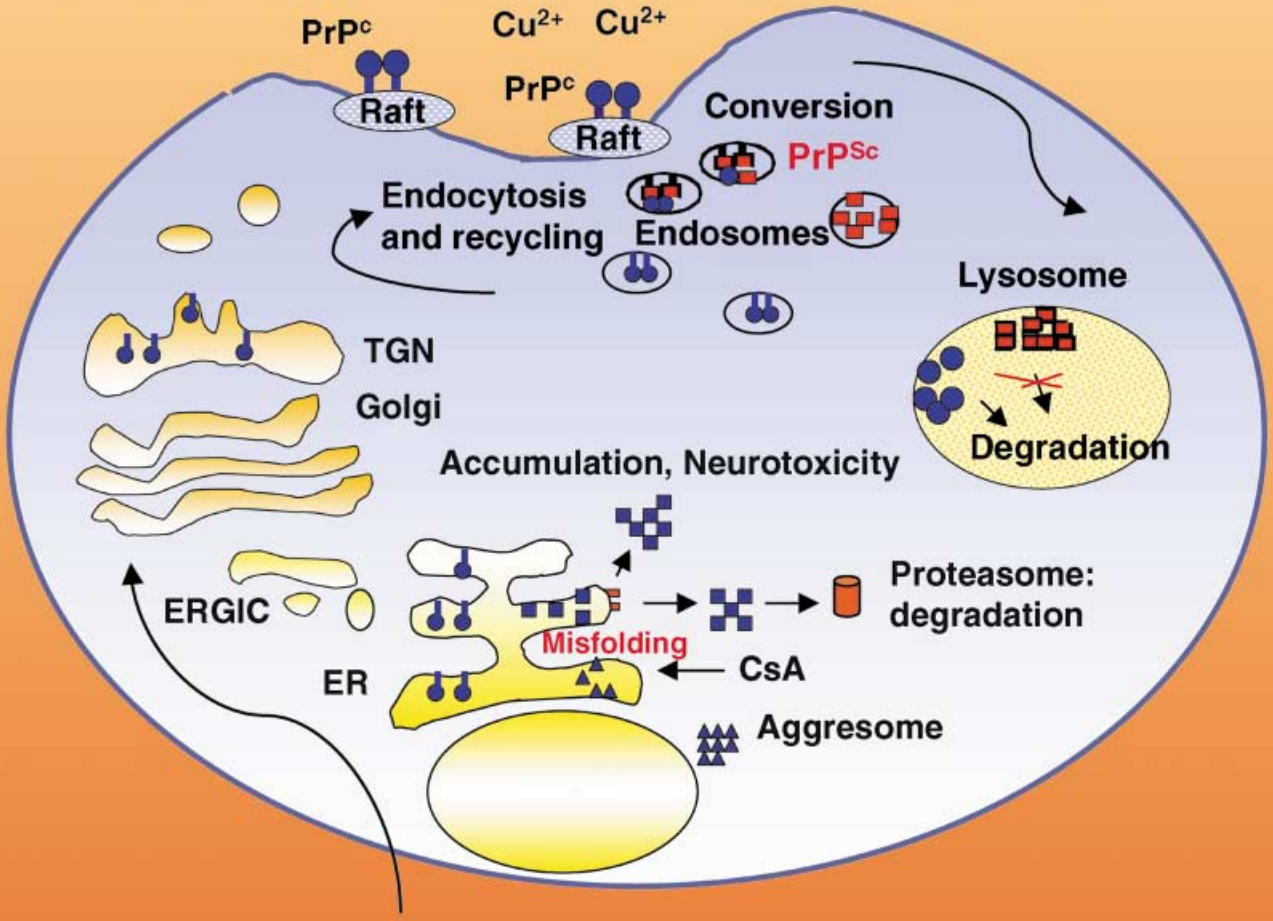
Prion Protein Trafficking



Understanding the molecular process of conformational conversion of the cellular prion protein is necessary for the development of therapeutic regimens against fatal neurodegenerative disorders such as vCJD.

Prion Diseases: From Molecular Biology to Intervention Strategies**

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Prion diseases are fatal neurodegenerative infectious disorders for which no therapeutic or prophylactic regimens exist. Understanding the molecular process of conformational conversion of the cellular prion protein (PrP^c) into its pathological isoform (PrP^{Sc}) will be necessary to devise effective antiprion strategies. In recent years, new findings in the cell biology of PrP^c, in the molecular pathogenesis of PrP^{Sc}, and in the cellular quality control mechanisms involved in these scenarios have accumulated. A function of the prion protein in signalling, the possible impact of the proteasome, and aggresomes as intracellular waste deposits have been described. Here, important pathogenetic similarities with the more frequent neurodegenerative disorders are evident. The need for therapeutic, postexposure, and prophylactic possibilities was drastically illustrated by the emergence of variant Creutzfeldt–Jakob disease (vCJD), a new human prion disease caused by bovine spongiform encephalopathy (BSE) derived prions. Although prion infectivity in humans is usually restricted to the central nervous system, in vCJD patients prions are present in the lymphoreticular system, posing a theoretical risk of accidental human-to-human transmission. A variety of chemical antiprion substances have been reported in in vitro and cell culture based assays or in animal studies. Occasionally, they have also made their way into the first human trials. In addition, various promising interference strategies have been devised in transgenic models, although they are usually hard to transfer into nontransgenic in vivo situations. New findings in the fields of peripheral prion pathogenesis and immune system involvement fuelled the search for antiprion strategies formerly considered to be entirely impossible. This opened the door towards classical immunological interference techniques. Remarkably, passive and even active vaccination approaches now seem to be realistic goals.

1. Introduction

Prion diseases, including Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle, are fatal and neurodegenerative infectious disorders. All of these diseases are characterised by the accumulation of PrP^{Sc}, the abnormally folded isoform of the cellular prion protein (PrP^c), which represents the major component of infectious prions.[1–3] The formation of PrP^{Sc} is accompanied by profound changes in the structure of PrP^c and its biochemical properties (Figure 1). PrP^c, which is rich in α-helical regions, is converted into a molecule with a mainly β-sheet structure. PrP^{Sc} is highly insoluble and partially resistant to proteolytic digestion.[1–5] During biogenesis, PrP^c is cotranslationally directed into the lumen of the endoplasmic reticulum by an N-terminal signal peptide that is 22 amino acids in length. This is removed, together with a C-terminal signal sequence of 23 amino acids, to promote attachment of a glycosylphosphatidylinositol (GPI) anchor. PrP^c undergoes further posttranslational modifications with the addition of two *N*-linked carbohydrate chains. Properly folded PrP^c transits through the secretory pathway and is attached to the outer leaflet of the plasma membrane by its GPI anchor.[6–8] Conversion of PrP^c into PrP^{Sc} seems to occur close to the plasma membrane along the endocytic pathway, probably in caveolae-like domains (CLDs) or in rafts, membranous domains or invaginations of the plasma membrane rich in cholesterol and glycosphingolipids.[9] Here, the first steps of PrP degradation occur[10, 11] before the protein reaches acidic compartments for final degradation. Cell surface localisation of PrP^c is thought to be essential for subsequent conversion into PrP^{Sc}[12–14] and studies in transgenic mice suggest a direct interaction between the two PrP isoforms, possibly in a complex with auxiliary factors,[1] either on the cell surface or in CLDs. The cellular function of the prion protein is still enigmatic, although binding of copper to the octapeptide repeat sequence located at its N terminus suggests a role of PrP^c in copper binding.[15, 16]

Prion diseases share important mechanistic aspects with the more frequent neurodegenerative diseases like Alzheimer's, Huntington's, and Parkinson's diseases. In sharp contrast to the

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[**] A list of abbreviations can be found at the end of the text.

latter disorders prion diseases are transmissible within and between species. In humans, three manifestions can be found (Table 1). Sporadic and familial Creutzfeldt – Jakob diseases both arise endogeneously, caused by a still-unknown ("sporadic") mechanism or by defined germline mutations in the prion protein gene (*Prnp*), respectively. The third form is the acquired prion diseases, which use known classical infection mechanisms (exogenous form). Although usually rare (sporadic CJD occurs worldwide with an incidence of 1 in 1 000 000), the infectious character makes these disorders extremely dangerous under certain conditions. In former times cannibalistic feasts might have contributed to epidemic situations. Recently, genetic evidence suggesting balanced selection and evolution of the *Prnp* gene against less susceptible populations has been reported.[17] However, this would indicate that cannibalism was more prevalent than previously thought.[17] A more recent example is kuru in Papua New Guinea which reached epidemic proportions in the forest people. In addition, BSE in its pandemic form and the rise of BSE-related diseases like vCJD have to be listed here.[18–20] Finally, various examples for smaller epidemics caused by iatrogenic transmissions of human prions are known. In particular, BSE has reinforced the transmissibility of prions to other species (for example, BSE to ungulates, carnivores, primates, and humans). Upon introduction into another species, prions often change various properties (such as tissue tropism) and enhanced intraspecies transmission can take place.[21] Chronic wasting disease (CWD) in deer and elk in North America represents another recent prion disease which shows an enormous increase in its incidence and which could be harmful to humans.[22, 23]

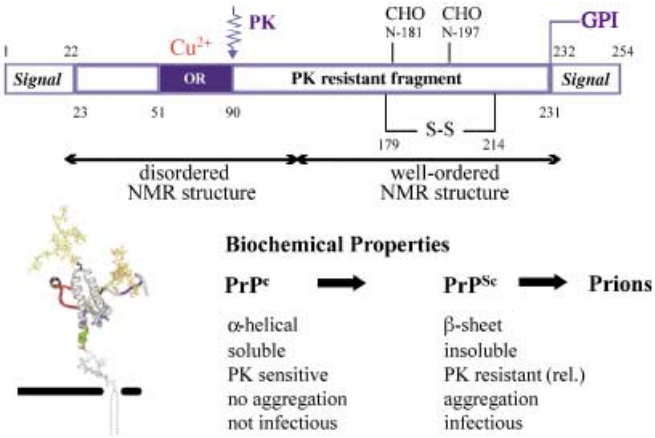

Figure 1. *Structural features and biochemical properties of prion proteins. The upper section shows the primary structure and posttranslational modifications. OR indicates the octarepeat sequence involved in copper binding. The lower left insert depicts a putative tertiary structure as deduced from NMR spectroscopy models.*

Table 1. *Manifestations of Prion Diseases.*

Manifestation	Disease	Mechanism
acquired	kuru, iatrogenic CJD, scrapie, BSE, feline spongiform encephalopathy (FSE), variant CJD (vCJD)	infection from outside, exogenous
sporadic	CJD (90%)	spontaneous change in PrP conformation (?), endogenous
genetic	CJD (10%), Gerstmann – Sträussler – Scheinker syndrome (GSS), fatal familiar insommnia (FFI)	germline mutation PrP, endogenous

2. Quality Control Mechanisms in Prion Pathogenesis

The metabolism of PrP^c after the protein has reached the cell surface and is localised in cholesterol-rich raft membranes has

Hermann M. Schätzl (right), born in 1962, studied medicine at the University of Munich. He received his MD in 1991 for work with retroviral oncogenes at the Max von Pettenkofer Institute in Munich, where he worked until 1992 on animal and human retroviruses. In 1993 he started experimental work with prions as a postdoctoral fellow in the laboratory of S. B. Prusiner at the University of California Medical School at San Francisco. He returned to the University of Munich in 1995 and moved to the Munich Gene Centre in 1996 as a principal investigator. Since 2002 he has held the Professorship for Clinical Virology at the Technical University of Munich. His group, together with S. Gilch and M. Nunziante, is currently engaged in molecular and cellular models for studying prion biogenesis and pathogenesis and in devising experimental antiprion strategies.

Sabine Gilch (center), born in 1971, started her experimental work with prions in 1996 as a specialist in the laboratory of H. M. Schätzl and is presently head of the prion laboratory.

Max Nunziante (left), born in 1969, studied biological sciences at the Universities of Padova (Italy) and Munich. He did his diploma thesis on immunological studies and skin grafting at the Institute of Histology in Verona (Italy), where he completed his Masters studies. Since 1999 he has been working in Munich under the supervision of H. M. Schätzl at the Gene Center and the Institute of Virology of the Technical University. He obtained his PhD in 2003 for work on the intracellular trafficking of the prion protein.

been delineated to a certain extent, with its degradation probably taking place in acidic compartments (Figure 2).[6, 8, 24] On the other hand, much less is known about the processing of the prion protein in the early compartments of the secretory pathway. In the recent past, a number of publications have brought new insight into the role of cellular quality control mechanisms and their possible implications in the pathogenesis of transmissible spongiform encephalopathies (TSEs).

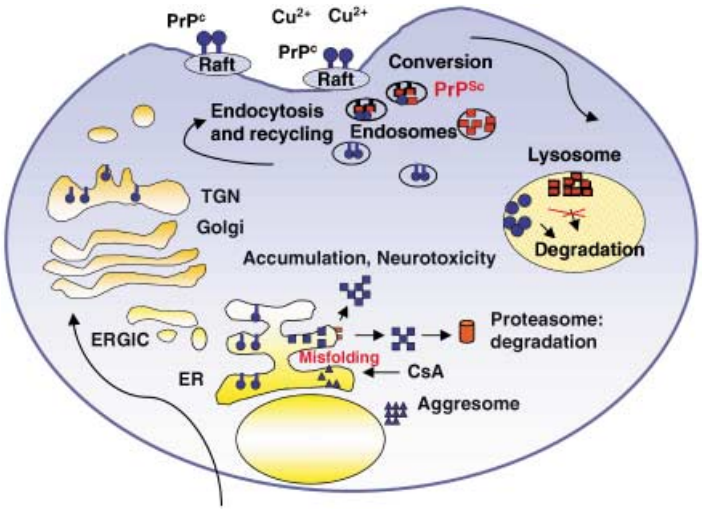

Figure 2. *Subcellular trafficking of the prion protein. Newly synthesised PrP^c (blue circles) is transported along the secretory pathway through the endoplasmic reticulum (ER) and the Golgi. At the cell surface, it is localised in cholesterol-rich domains (rafts or caveolae) by its GPI anchor. PrP^c is subjected to endocytosis and recycles to the cell surface or reaches lysosomes for final degradation. Conversion into PrP^{Sc} (red squares) occurs at the cell surface in rafts or in compartments of the early endocytic pathway. PrP^{Sc} is not efficiently degraded in lysosomes and accumulates. Misfolded PrP (blue squares) can be retrograde translocated into the cytosol and degraded by the proteasome or can accumulate and induce neurotoxicity. Treatment of cells with cyclosporin A (CsA) can induce aggregation of PrP molecules as aggresomes (blue triangles). ERGIC = ER – Golgi intermediate compartment, TGN = trans Golgi network.*

Several stringent quality control mechanisms operate in the cell to ensure that only newly synthesised proteins that have undergone correct co- and posttranslational processing and are properly folded are transported to their target organelles and cellular compartments. Incorrectly assembled proteins are normally retained in the ER and subjected to the ER-associated degradation (ERAD) pathway, which includes retrograde translocation through the ER membrane into the cytosol by the heterotrimer Sec61 complex (translocon).[25] The polypeptide chain is then deglycosylated by a cytosolic *N*-glycanase and the highly conserved protein ubiquitin is usually covalently bound to the lysine residues in the chain. Proteins are thereby marked for degradation by the 26S multiprotein proteasome complex.[26, 27] Although recent studies have described a lack of retrograde translocation into the cytosol and consequent accumulation of glycosylated substrates in the ER upon inhibition of the proteasome,[28–30] this retrograde transport is normally not dependent on an active proteasome.

Assumed to be of little relevance in the past, the roles of the proteasome and of the ER-related degradation pathway in the metabolism of the prion protein, as well as their implication in prion diseases, have become topics of research and debate in recent years. Previous studies performed with mutant prion proteins associated with GSS showed that these proteins are subjected to the ERAD pathway but, upon inhibition of the proteasomal activity, are partially retained in the ER and other membrane-bound compartments.[31–34] Although no involvement of ERAD in the metabolism of wild-type (wt) PrP^c had previously been assessed,[35] studies done under new experimental conditions have confirmed that, as is the case for other proteins passing though the ER, proteasomes and ubiquitin are associated with the turnover of the wild-type prion protein.[36, 37] Yedidia and colleagues showed that $\approx 10\,\%$ of the nascent wt PrP population was diverted into the ERAD pathway and accumulated in the cytosol in the presence of the proteasome inhibitors *N*-acetyl-leucinal-norleucinal, lactacystin, or MG132. These PrP molecules comprised detergent-soluble and -insoluble species. The insoluble aggregates were composed of unglycosylated PrP molecules which were partially resistant to proteinase K (PK) treatment, a feature usually associated with PrP^{Sc}, and included ubiquitylated PrP species.[36]

In parallel studies, the group of Lindquist confirmed the involvement of the ER-based quality control in the trafficking of the prion protein, as misfolded wt PrP was found to accumulate in the cytoplasm when proteasome activity was compromised upon treatment of cultured cells with several proteasomal inhibitors (MG132, lactacystin, and epoxomicin) having different chemical structures and mechanisms of action.[37] This PrP population seemed to have undergone the N- and C-terminal proteolytic cleavage events associated with normal processing in the ER and to colocalise with Hsc70, a member of the Hsp chaperone family that localises in the cytoplasm. It was therefore proposed that these molecules are delivered to the cytoplasm by retrograde transport from the ER into the cytosol. Longer treatments with inhibitors induced PrP to coalesce into a single large aggregate localised around the centrosome. Follow-up studies by the same group reported that, in cell culture experiments, the total amount of aggregated PrP present in the cytoplasm several hours after proteasomal activity had been restored exceeded the amount present immediately after inhibition.[38] These aggregated species presented properties normally associated with PrP^{Sc} (detergent-insolubility and partial PK resistance) and seemed to be able to sustain and promote misfolding of newly synthesised PrP^c even after reactivation of proteasomal activity. These results therefore claim that PrP has an inherent capacity to promote its own conformational conversion in mammalian cells and induces additional molecules to adopt the same conformation. Retrograde translocation of misfolded PrP, which could interact with other PrP molecules and promote conversion into a PrP^{Sc}-like conformation, was therefore proposed to be implicated in the initial steps of conversion in rare spontaneous PrP^{Sc} scenarios and in generating toxic PrP species. The described results find additional evidence in cell culture models and in vivo experiments performed on transgenic mice.[39] In these studies, the accumulation of even small

amounts of cytosolic prion protein resulted in extreme neurotoxicity. Transgenic mice expressing a mutant PrP lacking the N-terminal signal for ER translocation developed normally but acquired neurodegeneration with a pathology that was very similar to that seen in animals expressing mutant forms of PrP associated with human prion conditions.[40, 41] Since no PrP^{Sc} was detected in these mice, the described findings are in favour of an intrinsic toxicity of PrP^{c}. The authors of this study suggest that even small quantities of cytosolic PrP might act directly or through PrP cleavage products, possibly to activate cell death signalling pathways and induce neuronal damage. It is of note that almost no PrP^{Sc} accumulation is observed in several inherited and induced forms of prion disease.[42–45] According to this model, efficient ER quality control mechanisms normally shunt and degrade unfolded or misfolded PrP. On rare occasions, when aging takes place or due to biological traumas, the natural capacity of the system is compromised and, although misfolded PrP is still retrograde transported into the cytoplasm, degradation fails. In light of the described results, it was proposed that this process might lead to an increase in the amount of toxic cytosolic PrP or to the formation of new PrP^{Sc}. The cellular quality control could therefore play a significant role in the pathogenesis of familial and sporadic prion disease. Pathogenic mutations in the PrP coding sequence might also lead to an increase in misfolded PrP, recognition by the cellular quality control mechanism, and transport to the cytosol with consequent neurotoxicity. The authors point out that this mechanism might also explain pathogenesis in infectious prion diseases if extracellular PrP^{Sc} induces perturbations in the folding and trafficking of endogenous PrP. Despite the possible implications of these fascinating results in the field of prion diseases, the question remains of whether the effects described by Lindquist and co-workers are specific to the prion protein. Future studies on the consequence of cytosolic accumulation of proteins unrelated to the prion protein will be of great interest in the elucidation of the mechanisms of neurodegeneration.

Cohen and Taraboulos once more stated the importance of correct folding and of the cellular quality control mechanism in the metabolism of proteins in general and of the prion protein in particular in a recent publication.[46] In their study, the authors concentrated on the isomerisation of peptidyl prolyl bonds by the cyclophilins, a group of peptidyl prolyl isomerases (PPIases) expressed in most cellular compartments (including cyp A in the cytosol and cyp B in the ER).[47] Hampering the activity of the cyclophilin isomerases with the fungal immunosuppressant CsA in different cell lines led to accumulation of a PrP population with prion-like properties that was not ubiquitylated and partially resisted proteasomal degradation. Its distribution differed from the diffuse deposits monitored upon proteasomal inhibition. These aggregated PrP molecules formed aggresomes, perinuclear microtubule-dependent inclusion bodies located at the centrosome. Aggresomes characterise several neurodegenerative disorders related to toxic proteins[48–51] and were also described in the pathogenesis of known familial forms of prion disease.[52] These results argue for the ability of ER cyclophilins to isomerase the *cis* Xxx–Pro bonds that spontaneously form in a minority of the nascent PrP polypeptides to the native *trans* Xxx–Pro conformation. Inhibition of this PPIase activity of the cyclophilins with CsA therefore leads to accumulation of non-native *cis* Xxx–Pro peptides which, although retranslocated into the cytosol, are not proteasomally degraded. According to this model, the natural weakening of cyclophilin activity by aging could contribute to formation of the "prion seed" required for initiation of familial or sporadic prion diseases. This model also applies to two pathogenic mutations in the PrP gene linked to familial GSS and analysed in the study, P102L[53] and P105L.[54] These aberrant molecules, in previous studies characterised only by slight prion-like properties,[55] might be synthesised with *cis* Xxx–Leu bonds, which are less efficient substrates for cyclophilins and therefore remain incorrectly folded, and accumulate. Interestingly, clinical side effects of CsA have been described and include neurological signs such as ataxia.[56, 57] Chronic administration of this drug could result in cumulative effects. Whether protein misfolding and aggregation can contribute to these manifestations has not been elucidated. It is of note that aggresomes can impair the ubiquitin–proteasome pathway.[58]

An alternative explanation for the localisation of PrP in the cytosol is offered by Harris and co-workers who report that proteasomal inhibition upon over-expression of the prion protein in cultured neurons leads to inefficient translocation into the ER of a small fraction of the newly synthesised PrP population. These molecules were then rapidly degraded by the proteasome.[59] This group reports that treatment of cells with proteasomal inhibitors resulted in an increase in the level of PrP mRNA but had no effect on the maturation or turn-over of either wild-type or mutated PrP molecules. The cytosolic PrP molecules harbored an intact N-terminal signal peptide and lacked a GPI anchor and *N*-linked glycans, all features indicating that the protein has not undergone processing by signal peptidase, oligosaccharyl-transferase, or GPI transaminidase, all of which reside in the ER lumen. Such PrP chains remained closely associated with the cytoplasmic face of the ER. Following these findings, a model was proposed by this group in which failure in translocation of PrP, rather than retrograde translocation from the ER, accounts for accumulation of cytosolic PrP. Therefore, the accumulation of mature, unglycosylated forms of PrP upon long-term treatment with proteasomal inhibitors as described in the previously mentioned studies might, in part, be explained by the artificial increase in mRNA amount associated with over-expression levels in transfected cells. This model argues against a pathogenic role of cytosolic PrP and supports the idea that known forms of inherited prion disorders may rather be related to the toxic effects of misfolded PrP mutants accumulating in the lumen of the ER. Whether the severe neurodegeneration caused by cytosolic PrP is related to some forms of prion disease is therefore still a matter of debate. Nevertheless, in light of the possible damage to the nervous system caused by accumulation of PrP or by other misfolded proteins upon interference with proteasomal degradation, the use of proteasomal inhibitors in biochemical research and as therapeutic agents[60, 61] should be considered with care.

3. Signal Transduction Pathways and the Prion Protein

The search for therapeutic and prophylactic approaches for prion diseases goes hand in hand with the efforts undertaken in order to understand the biological function of the prion protein. This task is made harder by the fact that gene knock-out of *Prnp* in mice does not lead to a pronounced phenotype[62] and is associated with complete resistance to prion infection.[63] Several studies support a role of the cellular prion protein in copper metabolism[16, 64] with superoxide dismutase activity or as a carrier protein for uptake and delivery of metal ions from the extracellular to the intracellular environment.[65, 66] A function in maintenance and/or regulation of neuronal activity has been proposed, related to the abnormalities in synaptic physiology and circadian rhythm shown by some PrP-null mice strains.[67–69] Since the prion protein might also represent a cell surface receptor for a still unknown cellular or extracellular ligand, various biophysical assays were performed for identification of proteins interacting directly with PrP. First candidate interactors found were Bcl-2[70] and Hsp60.[71] A putative PrP receptor was identified by the group of Weiss.[72–74] Here, the 37 kDa laminin receptor precursor (LRP) and its mature 67 kDa form, termed the high affinity laminin receptor (LR), were seen to colocalise with PrP on the surface of murine neuroblastoma and BHK cells. Cell-binding assays revealed LRP/LR-dependent binding and internalisation of cellular PrP. The authors also suggest that this receptor might represent the portal of entry for PrP^{Sc} in prion infection.[73, 74] This study opens the question of whether LRP/LR – PrP interaction might contribute to cell-to-cell communication and might mediate intracellular signal transduction pathways. Recently, Mouillet-Richard et al. assessed the involvement of the prion protein in intracellular signalling adopting antibody-mediated cross-linking in a neuronal differentiation model.[75] A caveolin-1-dependent coupling of PrP^{c} to the tyrosine kinase Fyn was observed. In differentiated 1C11 cells, cross-linking of neuritic PrP^{c} induced a marked increase of Fyn kinase activity. Coimmunoprecititation assays identified caveolin-1 as one of the intermediate factors involved in the coupling of the intracellular Fyn to PrP^{c} anchored to the outer plasma membrane. The finding that PrP^{c} can be involved in intracellular signalling opens new directions for unravelling the function of this protein and implies its involvement in the modulation of neuronal function. Additional binding partners for the cellular prion protein were identified by using a yeast two-hybrid screen.[76] Given the fact that at least two of these PrP-interacting proteins, synapsin I and Grb2, are involved in neuronal signalling pathways, these data support the previous findings that one of the functions of PrP^{c} could be related to signal transduction pathways. Synapsins play an important role in the formation of synapses and in the regulation of neurotransmitter release,[77] and they are abundant in neuronal tissue and other cell types involved in exocytosis.[78] They provide a mechanism for controlling vesicle release by cross-linking vesicles to each other and to cytoskeletal proteins by a range of diverse kinases like calcium/calmodulin-dependent protein kinase I and protein kinase A. In cell-fractionation experiments, PrP^{c} and synapsin I colocalise in Golgi fractions[76] and PrP^{c} is strongly expressed in synaptic membranes,[79] a fact indicating that PrP^{c} might cooperate in the regulation of synaptic vesicles. It is of note that synapsin I is an interactor with the other protein identified in this screen, Grb2.[80] The main role of this adaptor protein seems to be the linking of signals coming from extracellular and/or transmembrane receptors, like the neuronal and epidermal growth factor receptors, to intracellular signalling molecules.[81] Since both of these proteins are of cytosolic origin, it was suggested that they might bind to one of the described transmembrane forms of the prion protein.[82] The proposed model of interaction of PrP^{c} with these two binding partners is a heterotrimer complex, as both synapsin I and Grb2 can bind to the N- and the C-terminal parts of PrP^{c}.[76] The function of such a complex could be related to the trafficking of vesicles and regulation of membrane fusion.

The identification of PrP^{c} as a signalling molecule opens new directions for exploring the elusive function of this protein whose activation might be triggered by extracellular signals. The question of how signal activity can be affected by PrP^{Sc} accumulation is therefore an important matter in the context of prion infection and for future therapeutic approaches against prion diseases.

4. The Subcellular Trafficking of the Prion Protein

The concern about the cellular function of the prion protein has led in recent years to a more accurate analysis of the metabolism and trafficking of this molecule and has increased interest in the possible role of specific segments in its polypeptide sequence. In particular, the N-terminal part of the prion protein containing the histidine-rich octapeptide repeat sequence (residues 51 – 90 in human PrP),[83] and its involvement in a cellular function have been a matter of intense study. In both the mainly α-helical PrP^{c} and in PrP^{Sc} with its high β-sheet content, the N terminus of the protein is devoid of a defined globular structure and it remains protease sensitive after prion conversion (Figure 1).[1, 5, 84, 85] Nevertheless, a comparative sequence analysis has evidenced a high conservation of the segment encompassing amino acids 23 – 90,[83] a finding that argues for a defined selective pressure for its conservation in evolution. In vivo studies with mice expressing N-terminally truncated PrP^{c} showed that deletion of residues 23 – 93 still supported prion propagation in these animals, although with reduced levels of detectable PrP^{Sc}, longer incubation times, and altered pathology.[86] These results were confirmed by in vitro cell-free conversion assays performed with hamster PrP^{c}, where deletion of amino acids 23 – 94 reduced the amount of protease-resistant PrP generated.[87] Other studies performed in cell cultures with N-terminally truncated PrP have led to the assumption that the N terminus of this molecule might stabilise the C-terminal domain or modulate binding to auxiliary factors participating in the conversion into the pathogenic isoform.[88] On the other hand, extra copies of the octapeptide repeats are associated with familial forms of CJD in humans[89] and neurodegeneration in transgenic mice.[90] Several recent studies have outlined that the physiological relevance of this segment might reside in the modulation of PrP internalisation. The finding that the histidine residues contained within the

octapeptide repeats represent binding sites for copper ions[64, 91] has led to the proposal that prion diseases might, in part, be related to abrogation of the normal cellular role of PrP^c in copper homeostasis.[92]

Binding of copper to the N terminus was also seen to affect the cell surface localisation of PrP^c by promoting endocytosis.[64, 93–95] In cell culture experiments, PrP^c was subjected to rapid endocytosis upon exposure of neuronal cells to physiological amounts of Cu^{2+} or Zn^{2+}, whereas deletion of four octarepeats abolished endocytosis.[95] Moreover, N-terminal deletion mutants previously shown to cause ataxia and neuronal degeneration[41] failed to internalise in response to Cu^{2+}.[94] These data therefore provide evidence that neurodegeneration associated with some forms of prion disease might arise from ablation of endocytosis promoted by metal ions due to mutations in the N-terminal part of the prion protein. The physiological relevance of this highly conserved region of the prion protein is also supported by more recent studies which, in line with previous findings,[96] define a more general subcellular sorting function for the segment encompassing residues 23 – 90.[97] In this work, the impaired intracellular trafficking and prolonged turn-over of PrP constructs presenting N-terminal deletions assess the importance of the entire N terminus of PrP as a targeting element in the transport to the cell surface, as well as in endocytosis. This function characterises not only mammalian species but applies also to more remote species and adds to the evidence that this segment might represent a binding element for a cellular transmembrane receptor. The relevance of the PrP^c N terminus is reinforced by new studies on the possible mechanism of internalisation and on the sorting of the cellular prion protein.[98, 99] In their accurate work, Sunyach et al.[98] confirm the importance of the N-terminal segment in endocytosis of PrP^c. The authors show that PrP^c is internalised in neuronal cells at a much faster rate than suggested by studies with transfected cell lines and that it leaves the detergent-insoluble rafts to cluster, together with other transmembrane proteins, in a fully soluble coated membrane environment. In this study, the N-proximal domain not only drives endocytosis but also determines the membrane localisation of PrP^c. The cluster of basic residues contained within this segment was shown to be of relevant importance. The targeting and association of PrP^c with sphingolipid- and cholesterol-rich rafts is once more explored in the work of Walmsley et al.[99] Here, transmembrane forms of PrP^c lacking the GPI anchor and expressed in human neuroblastoma cells failed to associate with raft membranes when the N-terminal domain (residues 23 – 90) was deleted. This region was sufficient to confirm raft localisation when fused to nonraft transmembrane and clathrin-coated pit proteins. Although contrary to previous reports which showed that transmembrane prion proteins failed to localise in lipid rafts,[9, 11] this report argues that the N-terminal region of PrP acts as a cellular raft-targeting determinant. Since these recent studies on PrP subcellular trafficking were conducted independently of Cu^{2+} binding (that is, no additional copper was required and Cu^{2+} chelators did not affect the outcome of the experiments), the relevance of the uptake of this cation by PrP in the metabolism of the prion protein still remains elusive.

5. Experimental Models for Therapy and Prophylaxis of Prion Diseases

An understanding of the molecular and cellular biogenesis and pathogenesis of PrP^c and PrP^{Sc} is needed for devising antiprion strategies. Although at the moment no therapeutic or prophylactic regimens are available for prion disease in patients, many substances have been tested in different experimental models for their antiprion activity. These models can be roughly divided into three categories. First of all, there are the mere in vitro systems which use the possibility of cell-free in vitro conversion if PrP^c is incubated under certain conditions with PrP^{Sc} derived from prion-infected brain homogenates.[100] It is of note that, although PK-resistant prion protein (termed PrPres) is generated, this system fails to demonstrate the de novo generation of prion infectivity in bioassays.[101] In another in vitro model, prion infected cells which persistently propagate infectious prions[102, 103] are used for the screening of various classes of substances. As a read-out, the decrease of PrP^{Sc} in treated cells is measured, either by biochemical tools (PK treatment and/or solubility assay for PrP^{Sc} followed by immunoblotting or immunoprecipitation) or by monitoring the decrease of prion infectivity in indicator bioassays (Figure 3). For the latter, cell

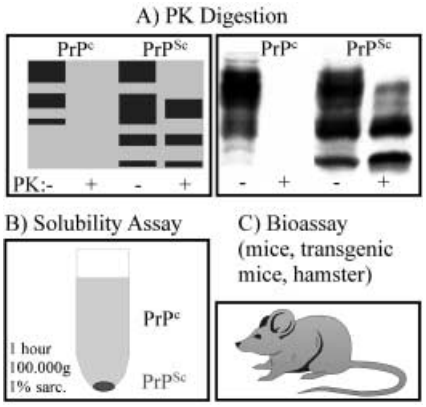

Figure 3. *Experimental possibilities for discrimination of PrP^c and PrP^{Sc}. A) Figure depicting the relative PK resistance of PrP^{Sc}. Lysates of cells or tissues are digested with PK under standard conditions (usually 20 – 40 μg of PK per mL for 30 – 60 min at 37 °C) and analysed by immunoblotting. PrP^c is completely sensitive to PK digestion; PrP^{Sc} is N-terminally truncated (PrP 27 – 30; digestion of residues ≈ 23 – 90). In murine prion-infected cells and tissues the typical three-banding pattern of PrP^{Sc} (from un-, mono-, and diglycosylated PrP) results. B) Another possibility for biochemical differentiation is based on the insolubility of PrP^{Sc} in nonionic detergent (for example, 1% sarcosyl). The lysate is suspended with 1% sarcosyl (sarc.) and ultracentrifuged for 1 h at 4 °C and 100 000 g. Pellet and supernatant fractions are analysed by immunoblotting. PrP^c stays soluble in the supernatant and PrP^{Sc} is found insoluble in the pellet. C) Specific prion infectivity can only be tested in bioassays.*

lysates are injected intracerebrally into susceptible, in most cases transgenic, PrP-overexpressing mice, and the incubation time to clinical prion disease is measured. Finally, animal models (for example, mice, Syrian hamsters, transgenic mice) allow the investigation of prophylactic and therapeutic effects in the context of a whole organism. Not only can antiprion effects be measured but also possible side effects and, most importantly,

the ability of substances to cross the blood – brain barrier (BBB). As a special condition, mutated prion proteins acting as transdominant negative inhibitors when expressed on the PrP wild-type background can be investigated in transgenic mouse models, thereby opening the door for gene therapy approaches.

6. Therapy and Prophylaxis in Prion Diseases: A Realistic Goal?

Despite some obvious experimental interference possibilities, one has to ask whether therapy and/or prophylaxis against prion diseases are realistic goals. Human prion diseases have a very long incubation time (years to decades), a short clinical phase (months), and no preclinical diagnosis; the disease is always fatal and the brain is massively damaged. Therefore, when symptoms manifest, it seems to be a point of no return and too late for therapy. A tremendous problem, as is the case with all neurodegenerative disorders, is that therapy needs delivery of antiprion compounds directly to the central nervous system (CNS), which means crossing the blood – brain barrier (CNS therapy, Table 2). It is of note that there are examples for prion diseases where infection occurs from peripheral sites of the body (for example, oral and intraperitoneal infection) and where propagation and transport of prions outside the CNS have to take place before neuroinvasion can occur. In such situations extra-CNS therapy and/or prophylaxis can be applicable, and even postexposure prophylaxis might be conceivable, given the very long incubation times. In particular, for vCJD, for which future case numbers are unknown[104] and where the risk of secondary human-to-human transmission by iatrogenic routes exists,[105] prophylactic tools are urgently needed. A new risk factor has also shown up. Recent publications have indicated that muscle tissue can harbor significant levels of infectious prions.[106, 107]

Table 2. *Pathogenesis of prion disorders and theoretical possibilities for therapeutic and/or prophylactic intervention.*

Disease	Acquired[a]	Treatment[b] CNS/extra-CNS	Prophylaxis[c] CNS/extra-CNS
sporadic CJD	no	CNS only	not known
familiar CJD/GSS	no	CNS only	conceivable although CNS
iatrogenic CJD[d]	yes	extra-CNS, if early	extra-CNS conceivable[d]
kuru[e]	yes	extra-CNS, if early	extra-CNS conceivable
vCJD[e]	yes	extra-CNS, if early	extra-CNS conceivable
BSE[e]	yes	extra-CNS, if early	extra-CNS conceivable
scrapie[e,f]	yes	extra-CNS, if early	extra-CNS conceivable
CWD[e,f]	yes	extra-CNS, if early	extra-CNS conceivable

[a] Pathomechanism of human prion diseases: endogenous (sporadic/familiar) versus exogenous (acquired, for example, by peripheral infection); for sporadic CJD no preclinical diagnosis is available, for familiar CJD/GSS/FFI preclinical diagnosis is possible (DNA test, family history). [b] Treatment of CNS versus extra-CNS treatment, with or without crossing the blood – brain barrier; extra-CNS = peripheral nervous system and/or lymphoreticular system; postexposure prophylaxis is possible if the time point of inoculation known. [c] Prophylaxis, for example, interference with peripheral propagation and transport, abrogation of neuroinvasion (prevention of CNS disease). [d] Iatrogenic CJD can be acquired by peripheral (for example, human growth hormone) and central routes (such as dura mater transplantation). [e] Peripheral infection (for example, oral route). [f] Scrapie and CWD have both acquired (horizontal/vertical transmission) and genetic/sporadic characteristics.

7. Chemical Substances Interfering with Prion Propagation

The first line of antiprion approaches uses chemical compounds known in most cases from other applications[108] (summarised in Table 3). Cyclic tetrapyrroles like hemes and chlorophylls are known to be a class of compounds that bind selectively to proteins and induce conformational changes.[109, 110] The central ring structure is important for the interaction with proteins. Different molecules out of this group have been tested in a cell-free in vitro conversion assay and have been shown to inhibit the formation of PrPres.[111] The most potent inhibitors, phthalocyanine tetrasulfonate (PcTS; Figure 4A) and the porphyrins *meso*-tetra(4-*N*-methylpyridyl)porphine iron(III) (TMPP-Fe^{3+}) and deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron(III) (DPG_2-Fe^{3+}) were further investigated in bioassays employing mice overexpressing hamster PrP.[112] If applied 0 – 4 weeks (12 treatments in total) after intraperitoneal (i.p.) infection, survival time was increased by 50 – 300 %. (Up to 300 % in the case of PcTS.) This application scheme reflects a typical experimental postexposure prophylaxis situation. In a more therapeutic approach, the drugs were applied 28 or 56 days after infection (dpi). Only PcTS delayed the onset of prion disease when treatment started 28 dpi, a finding that suggests a more prophylactic mode of action of tetrapyrroles. Mechanistically, PcTS seems to inactivate prions by direct interaction with PrP^{Sc}. Cyclic tetrapyrroles inhibited both hamster and mouse PrPres formation in in vitro conversion assays, thereby indicating that binding to prions is not strain specific. In contrast, for another group of antiprion agents some strain specificity was shown. This class comprises branched polyamines.[113, 114] Several preparations of branched polyamines,

Table 3. *Compounds exhibiting therapeutic and/or prophylactic antiprion effects.*[a]

Class of compounds	Example
polysulfonated, polyanionic substances	congo red
	dextran sulfate
	pentosan polysulfate
polyene antibiotics	amphotericin B
	MS-8209
anthracyclines	4-iodo-4-deoxy-doxorubicin
designer peptides	β-sheet breaker
cyclic tetrapyrroles	porphyrines, phthalocyanines
polyamines	SuperFect, DOSPA
immune modulators	soluble lymphotoxin-β (LTβ) receptor
inducers of aggregation	suramin
acridines/bis-acridines	quinacrine
phenothiazines	chlorpromazine
aptamers	RNA aptamer DP7
antibodies	monoclonal anti-PrP antibodies
	recombinant anti-PrP Fab fragments
	polyclonal anti-PrP auto-antibodies

[a] Tested in cell cultures and/or bioassays.

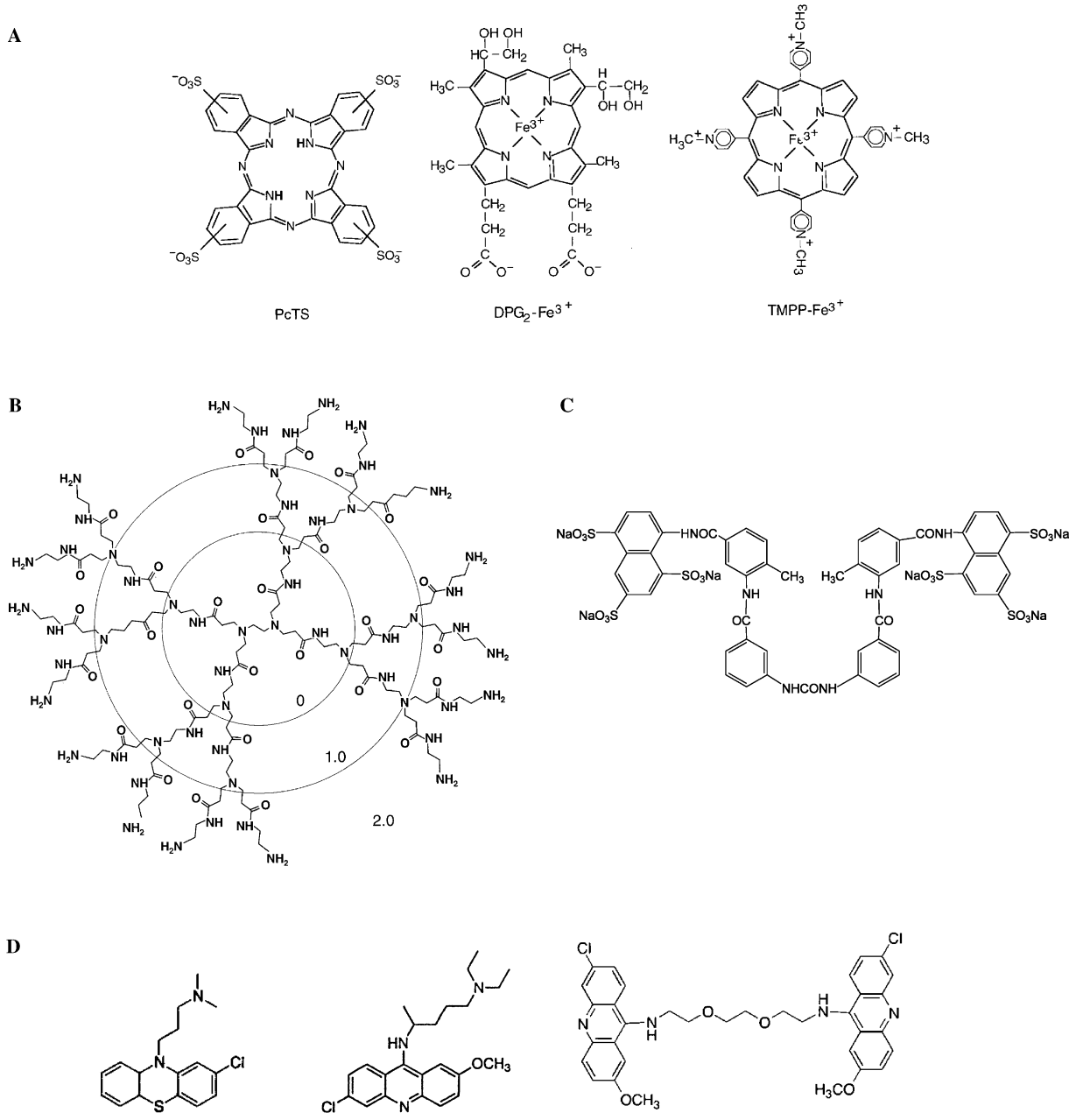

Figure 4. *Chemical structures of various antiprion compounds. A) The phthalocyanine PcTS (left panel) and the two porphyrins DPG$_2$-Fe^{3+} (middle panel) and TMPP-Fe^{3+} (left panel). B) Schematic diagram of the polyamidoamide (PAMAM) dendrimer. C) Formula of the polysulfonated naphthyl urea compound suramin. D) Representatives of the different acridines and phenothiazines are shown: Chlorpromazine (left panel), quinacrine (middle panel), and the most effective bis-acridine (right panel), linked by an alkyl ether linker.*

including PAMAM (Figure 4B), polyethyleneimine, and polypropyleneimine dendrimers, were tested on prion-infected neuroblastoma cells (ScN2a) at nontoxic concentrations for their ability to interfere with PrP^{Sc} propagation. All these compounds eliminated PrP^{Sc}. Increasing the molecular size of the dendrimers and the density of primary amino groups at the surface correlated with an increase in antiprion activity. One hallmark of PrP^{Sc} is its partial resistance to proteolytic digestion and its accumulation in the cells and brains of diseased organisms. In prion-infected cells, it has a half-life time of more than 24 h.[6] Treatment of ScN2a cells with branched polyamines enabled the cells to degrade PrP^{Sc} with a decreased half-life time of $\approx$4 h. Moreover, the cells seemed to be cured from prion infection, since PrP^{Sc} did not reappear after withdrawal of the compounds and further cultivation. In indicator bioassays with lysates of polypropyleneimine-treated cells the 50% infective dose (ID_{50}) was reduced more than 10000-fold. In addition, the role of an acidic pH environment was confirmed by in vitro degradation assays, where prion-infected mouse brain homogenate was mixed with branched polyamines at different pH values and subsequently digested with PK. Only at pH 4.0 or below, was sensitivity to PK digestion induced. In further studies, the

induction of PK sensitivity was shown to be dependent on the primary structure of PrP and the prion strain.[114] Although PrP is a protein with a primary structure that is highly conserved between different species,[83, 115] amino acid exchanges at certain positions cause the species-barrier phenomenon, which inhibits transmission of prions from a certain species to another. A prominent example of this is the failure to clinically infect mice with hamster prions.[116] In addition, prion strains are known that can share the same primary structure, but have different glycosylation patterns and conformations, and target different regions in the brain.[117–119] In in vitro degradation assays, branched polyamines induced strain-specific (several mouse strains were analysed) and species-specific (for example, degradation of mouse or hamster prions of the same strain) disaggregation, reduction of β-sheet content, and finally susceptibility to PK digestion of prions. Although these studies lack direct evidence for prophylactic or therapeutic benefits, for example, by treatment of infected animals with branched polyamines, the strain specificity offers new possibilities for diagnostic purposes and strain typing. Similar results in cell cultures were obtained when the cationic lipopolyamine DOSPA was added to the medium of ScN2a cells.[120] DOSPA, like the branched polyamines, induced cellular degradation of pre-existing PrP^{Sc}. Reduction of PrP^{Sc} was specific for lipids containing a headgroup of the polyamine spermine and a quarternary ammonium ion between the headgroup and the lipophilic tail. Treatment did not affect the biosynthesis of PrP^{c} or the assembly of cholesterol-rich microdomains/rafts at the plasma membrane, which are the proposed site for prion conversion.[9] PrP^{Sc} propagation proceeded after withdrawal of the substance, although at a lower level, a finding that suggests a less pronounced effect of PrP^{Sc} degradation than the one achieved with branched polyamines.

For the molecular conversion of PrP^{c} into pathological PrP^{Sc} two models exist. In the template-assisted model (Figure 5), PrP^{c} forms a heterodimer with PrP^{Sc}, possibly in complex with auxiliary factors, for example, protein X. The so-far unidentified protein X is expected to interact with PrP^{c} by using a discontinuous epitope at the C-terminal part of PrP,[121, 122] mainly

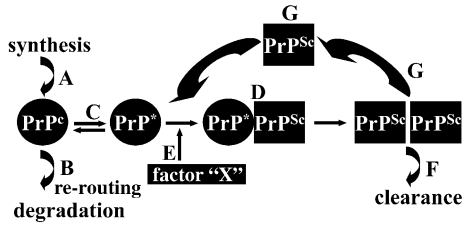

Figure 5. *Model of prion conversion and various potential possibilities for interference. A: Blocking PrP^{c} synthesis (e.g. $PrP^{-/-}$ mice); B: intracellular re-routing of PrP^{c} (e.g. suramin); C: overstabilising PrP^{c} (e.g. chemical chaperones, Congo red); D: interfering with interaction of PrP^{c} and PrP^{Sc} (e.g. β-sheet breaker, anti-PrP antibodies/monoclonal antibodies (mAbs)/fragment antigen binding (Fab), PrP-Fc_2); E: interfering with binding of putative additional components (factor X) involved in prion conversion (e.g. PrP-Fc_2); F: increase of cellular clearance of PrP^{Sc} (e.g. polyamines, anti-PrP antibodies/mAbs/Fab); G: sequestering incoming and nascent PrP^{Sc} (e.g. PrP-Fc_2). Several simultaneous interfering possibilities by a single compound are possible.*

involving four amino acid residues.[88, 122] PrP with a mutated protein X binding site was not converted into PrP^{Sc} when expressed in transgenic mice. Moreover, the conversion of endogenous wt PrP was prevented.[88] Therefore, protein X offers a site for potent inhibition of PrP^{Sc} biosynthesis (Figure 5). In order to mimic this transdominant negative effect on PrP^{Sc} biogenesis, a computational search was conducted on the Available Chemicals Directory for small heterocyclic molecules that copy the spatial orientation and basic polymorphism of the protein X binding site.[123] Sixty-three compounds were tested on ScN2a cells and two compounds, 2-amino-6-[(2-amiophenyl)-thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile (Cp-60) and *N*′-1-({5-[(4,5-dichloro-1*H*-imidazol-1-yl)-*m*-ethyl]-2-furyl}carbonyl)-4-methoxybenzene-1-sulfonohydrazide (Cp-62), dose-dependently inhibited PrP^{Sc} formation at nontoxic concentrations. Searching for substructures of the most effective compound Cp-60 identified three molecules with an activity comparable to Cp-60. Determination of the concentration at which 50% of PrP^{Sc} replication was inhibited (IC_{50}) revealed relatively high values of 18 – 60 μM. Two out of the five compounds were toxic to the cells at such concentrations. No further analysis of these compounds in bioassays is provided, and the in vitro data suggest a daily dosing schedule in this system. Direct evidence for interference with protein X binding to PrP^{c} is lacking, therefore it is difficult to assess at the moment whether drugs identified by computational analysis really specifically recognise the desired target.

According to the seeded aggregation model for prion conversion suggested by Lansbury and co-workers, spontaneously formed PrP^{Sc} acts as a crystal seed for further addition of PrP^{Sc} molecules, and large PrP^{Sc} aggregates are formed (see Figure 6).[124] Recently, nuclease-resistant 2′-amino-2′-deoxypyrimidine-modified RNA aptamers that recognise with high specificity a peptide comprising amino acid residues 90 – 129 of the human prion protein were selected by screening a combinatorial library.[125] This domain of PrP is thought to be functionally important for the conversion of PrP^{c} into PrP^{Sc} and is highly homologous among prion proteins of various species including mouse, hamster, and man.[83, 115] The most promising candidate, aptamer DP7, binds to the full-length human, mouse, and hamster PrP in vitro. At low concentrations (700 nM) in the growth medium of ScN2a cells, aptamer DP7 significantly reduced the relative proportion of de novo synthesised PK-resistant PrP^{Sc} within only 16 h. Mechanistically, DP7 might be incorporated into PrP^{Sc} aggregates and prevent the formation of high-molecular-weight aggregates, thereby mediating susceptibility to proteolytic digestion (Figure 6). RNA aptamers provide a novel class of antiprion compounds with an IC_{50} below 1 μM, without inducing cytotoxicity, and exhibiting strong effects when added to the cells for only a short period of time. Their effectiveness in bioassays remains to be determined, but the ability of RNA aptamers to cross the blood – brain barrier is unlikely. Notably, synthesis of nuclease-resistant RNA aptamers is very expensive, which makes them unattractive for high-dose in vivo application.

The substances described above were shown to be effective in cell cultures or in in vitro conversion assays, and in part they

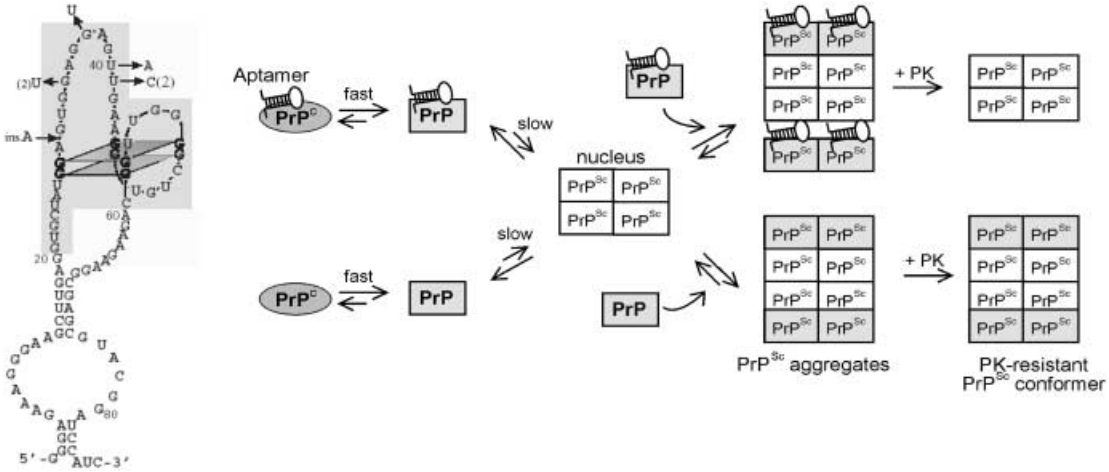

Figure 6. *Antiprion RNA aptamers.[125] Sequence and proposed structure of aptamer DP7 (left). Model for the mechanism of DP7-specific reduction of PrP^{Sc} formation based on the nucleation-dependent polymerisation model.[124] Aptamer DP7 binds to surface-located PrP^{c} and remains bound to PrP. The de novo formation of high-molecular-weight, tightly folded PrP^{Sc} aggregates originating on a preexisting crystal seed is modulated in the presence of the aptamer. Directly connected with aggregate formation and tight folding is the acquisition of PK resistance. In the event of aptamer binding, PK resistance is reduced.*

were tested successfully in animal models without inducing severe side effects. Nevertheless, it is unpredictable whether these drugs can be used in humans due to their unknown pharmacokinetic behaviour. Most of them are not able to cross the BBB, which restricts their application to prophylactic or postexposure scenarios. Therefore, several groups focussed their attention on drugs which can cross the BBB and/or have been used already in humans for medical application. One compound which was used for decades in humans to treat trypanosomiasis is the polysulfonated naphthyl urea compound suramin (Figure 4C).[126] When applied to ScN2a or ScGTI cells,[102, 103] de novo synthesis of PrP^{Sc} was completely inhibited, and after removing the drug and further cultivating the cells, the PrP^{Sc} signal did not reappear. Suramin is known to interfere with aggregation and surface expression of certain proteins, and in the case of PrP^{c}, it induced the formation of insoluble full-length PrP aggregates, which were, in contrast to PrP^{Sc}, entirely sensitive to proteolytic digestion. In this study, a novel mechanism for the inhibition of prion conversion was established. The misfolded PrP aggregates were recognised by a cellular quality control mechanism residing in post-ER compartments. Consequently, PrP aggregates were directly rerouted from the Golgi/trans Golgi network to lysosomes for degradation, without reaching the plasma membrane. Thereby, the compartment of prion conversion was by-passed (Figure 7). In mouse bioassays, i.p. application of suramin around the time point of peripheral prion inoculation significantly prolonged the onset of prion disease. In this study, an important example is provided which shows that mechanistic data obtained in cell culture can be directly transferred to in vivo situations. Unfortunately, suramin does not cross the BBB, but derivatives of suramin are currently under investigation (Nunziante et al., unpublished data).

The group of Prusiner tested various acridine and phenothiazine derivatives for their inhibitory effect in cell cultures.[127] Phenothiazines are compounds with a tricyclic scaffold and a side chain extending from the middle ring moiety (Figure 4D).

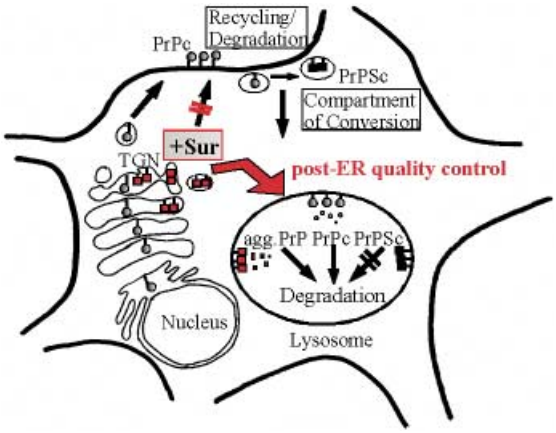

Figure 7. *Model of suramin (Sur) effects on PrP biogenesis and propagation of PrP^{Sc}.[126] The biosynthesis of PrP^{c} in the exocytotic pathway and its recycling and degradation in the endocytotic pathway are depicted (PrP^{c} shown by shaded circles). The putative subcellular compartment of conversion of PrP^{c} into PrP^{Sc} in the early endocytotic pathway is marked. PrP^{Sc} is indicated by black boxes. The induction of detergent-insoluble PrP aggregates by suramin in post-ER compartments and the direct re-routing to acidic vesicles is indicated (+ Sur, red arrow, PrP depicted by red boxes). By this rerouting the plasma membrane localisation and the putative compartment of prion conversion are by-passed.*

Among these are the antimalarial drug quinacrine and the antipsychotic compound chlorpromazine. Both drugs have been used in humans for nearly 50 years, and they are known to penetrate the BBB. In cell cultures, they proved to be effective against PrP^{Sc} formation, with rather low IC_{50} values of 3 μM for chlorpromazine and only 0.3 μM in case of quinacrine (previously described in ref. [128]). These encouraging results led to the immediate start of clinical trials with quinacrine for humans suffering from CJD or vCJD. So far no beneficial effect exerted by the quinacrine treatment has been published. Furthermore, several patients needed to stop taking the drug due to severe

liver damage and other side effects caused by high-dose administration of quinacrine.[129] In the meantime, quinacrine was tested in a mouse-adapted CJD model. Mice were infected intracerebrally and after different time points treatment was started by oral application. Unfortunately, quinacrine did not prolong the survival time of the mice.[130] Studies based on quinacrine in cell cultures were extended, and in order to find compounds with a better effect on PrP^{Sc} propagation, covalently linked dimers of quinacrine were tested (Figure 4D).[131] Bisacridines that are ten times more effective than quinacrine were identified and the importance of linker length and structure was revealed. It will be interesting to see whether these derivatives are effective against prion disease in bioassays.

In summary, many classes of chemical compounds have been identified with pronounced effects on prion propagation in vitro and in vivo. Most of them do not cross the BBB and this excludes them from use in therapy (Table 2). Application directly into the CNS or cerebrospinal fluid, the search for effective derivatives of known compounds, and pharmaceutical developments which enable the packaging of drugs to allow the penetration of the BBB are highly desirable.

8. Approaches Involving Gene Therapy

Based on the above-mentioned interference with prion propagation by targeting the putative protein X binding site, two out of four residues of the epitope mapped for protein X binding were mutated (Q167R and Q218K) and expressed in transgenic mice.[132] These mutant proteins were shown previously to act as transdominant inhibitors when transfected into ScN2a cells.[88] Naturally occurring polymorphic variants of the corresponding residues in sheep or humans are known to render them resistant to scrapie and CJD, respectively.[133–135] Transgenic mice expressing these mutant prion proteins on either a $PrP^{0/0}$ or wild-type background were inoculated with prions. The expression of the mutated PrP significantly slowed endogenous PrP^{Sc} formation. Moreover, dominant-negative PrP mutants were not converted into PrP^{Sc}. Given these findings and the knowledge that these mutations are naturally occurring polymorphisms that mediate resistance to prion disease, gene therapy could become a useful tool in prion disease. Nevertheless, this requires first the improvement of the vehicles used for the delivery of recombinant DNA in general.

Recently, a study employing transgenic mice to characterise the effects of a soluble immunogluobulin G (IgG) like dimeric PrP on prion propagation was presented.[136] The investigated molecule is a dimer consisting of two PrP moieties fused to the Fcγ tail of human IgG_1 and is designated PrP-Fc_2. Mice expressing PrP-Fc_2 on a $PrP^{0/0}$ background were created and then inoculated with prions by the i.p. or intracerebral (i.c.) route. All mice remained healthy, a finding indicating that PrP-Fc_2 is not convertible into PrP^{Sc}. In animals expressing PrP-Fc_2 on a $PrP^{+/+}$ background, the incubation time for prion disease was significantly prolonged and was dependent on dose and route of infection. In summary, PrP-Fc_2 competitively inhibits, but does not completely block prion accumulation in the CNS and the lymphoreticular system.

In the investigation into the molecular basis for inhibition of prion propagation in the presence of PrP-Fc_2, a great body of evidence points to an interaction between PrP-Fc_2 and PrP^{Sc}, either by direct binding or in a complex with further proteins. Complex formation seems to occur in lipid rafts, the postulated site of prion conversion. Thereby, the interaction between PrP^{Sc} and PrP^{c} or with other proteins necessary for prion synthesis might be prevented (Figure 5). A novel transgene, namely a soluble dimeric IgG-like PrP which cannot be converted into PrP^{Sc}, and which inhibits prion propagation when coexpressed with wt PrP, was described. The beauty and promising novel aspect of PrP-Fc_2 is that it does not depend entirely on gene therapy approaches. It could be expressed in mammalian cells in a soluble form and purified at high amounts from the culture medium. This would provide a powerful new tool for postexposure prophylaxis against acquired forms of prion disease, as the inhibitory effect on peripheral prion accumulation has been proven. As a note of caution, it should be kept in mind that injection of a foreign molecule like PrP-Fc_2 into immunocompetent recipients might cause severe side effects or at least lead to an immune response, which would eventually result in the rapid degradation of PrP-Fc_2.

9. Prion Disease and the Immune System

Prion diseases are a group of disorders that do not cause an immunological response. In brains, no inflammatory reaction is detectable and peripheral prion infection does not result in antibody production. One explanation is the obvious autotolerance to the self-protein PrP.[137] Recent work has pointed to the pivotal role of components of the immune system in prion infection from peripheral sites. This has been shown in various transgenic mice, impaired, for example, in B-cell maturation, follicular dendritic cell (FDC) maturation, or complement factors.[138–142] These findings can be of use for approaches to prevent the peripheral propagation and the transport of prions to the CNS, which is necessary after peripheral infection, for example, in vCJD (Table 2). FDCs, which are resident cells in the spleen and in lymph nodes, are an important site for peripheral prion propagation.[140, 142] For FDC maturation, tumour necrosis factor and LT are essential.[143–145] Inhibition of the LTα/β pathway with an LTβ-receptor – immunoglobulin fusion protein (LTβR-Ig) leads to the disappearance of functional FDCs. The effect of LTβR-Ig treatment on the pathogenesis of prion diseases was studied in mouse bioassays.[146] Treatment started one week before or one week after i.p. infection and was maintained until seven weeks after inoculation. Analysis of spleens at different time points confirmed the absence of FDCs, and no or only traces of PrP^{Sc} were detectable. Neuroinvasion was delayed, although this was more pronounced if treatment started one week before inoculation. After LTβR-Ig administration was terminated, the FDC networks in the spleen were reconstituted. As vCJD affects the lymphoreticular system, treatment with LTβR-Ig might be feasible if early diagnosis is possible.

The role of the cells of the innate immune system, like macrophages, monocytes, and dendritic cells, in prion disease is

rather unclear. Splenic macrophages seem to be involved in the clearance of PrP^{Sc} after peripheral infection,[147] but overall, no detectable stimulation of the innate immune system is induced by prion infection. Since prions are thought to consist solely of protein without containing immunostimulatory nucleic acids, Sethi and co-workers[148] tested whether administration of CpG oligodeoxynucleotides (ODN) influences the incubation time of prion disease after i.p. inoculation. CpG motifs, occurring with a high frequency in bacterial DNA, contain a central unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines. They stimulate the innate immune system by inducing a signalling cascade through the TLR9 receptor to result in cytokine production and immune cell proliferation.[149] Mice were i.p. infected and CpG ODN were applied at 0 h postinfection and daily for 4 days (group 1), or after 7 h postinfection and then daily for 4 days (group 2) or 20 days (group 3). Groups 1 and 2 survived 38% longer than the control mice, and in group 3 incubation time was >330 days (control mice died after between 181 and 183 days). CpG ODN were applied very frequently and at a high dose, and it is not clear whether the effect is specific for the activation of innate immunity. First, it might be due to an adjuvant effect of CpG that results in antibody production. Second, repeated stimulation with high doses of CpG ODN might heavily impair the architecture of the spleen by overstimulation of immune cells, thereby resulting, for example, in a functional lack of FDCs. Furthermore, it cannot be assumed that the effect seen in mice can be transferred to the human situation, for example, because of a different distribution of the TLR9 receptor on innate immune cells. Similar results were obtained in a study where complete Freund's adjuvant (CFA) was used as an adjuvant for prion peptide immunisation.[150] After i.p. inoculation the survival time of the mice was significantly prolonged. Unexpectedly, there was no difference in the incubation times between mice injected with prion peptide and CFA and the control group inoculated with CFA alone. Although the effect was less pronounced than the one described for CpG ODN, this work again indicates that the innate immune system might exert a protective role in prion disease.

In the last two years, several studies have pointed to beneficial effects of antibodies as antiprion compounds in transgenic mouse models[151] and in prion-infected cells.[152, 153] Treatment of ScN2a cells with the monoclonal antibody 6H4 dose-dependently abolished PrP^{Sc}.[152] Infection of N2a cells with brain homogenate was inhibited by treatment with 6H4. In a second study, different Fab fragments were examined for their ability to inhibit prion propagation in ScN2a cells.[153] Several effective Fabs were identified, with Fab D18 being the most effective. Indicator bioassays with treated cells confirmed that not only PrP^{Sc} but also prion infectivity was significantly reduced by Fab treatment. The high potency of D18 compared to the other Fabs was explained by its capacity to bind a significantly greater number of cell-surface PrP^{c} molecules and by the epitope recognised. This epitope comprises residues 132–156 and is spatially positioned on the opposite side to the proposed protein X binding site. Therefore interaction with protein X might be disturbed. It is of note that the 6H4 antibody used by Enari et al. covers a similar epitope (amino acids 144–152) to D18.[152] The disadvantage of Fab fragments for in vivo application is their short half-life, therefore whole antibodies might be much more useful.

In a study employing a transgenic approach, mice with a skewed endogenous IgM/D repertoire were designed, and expression of one type of IgM was reintroduced by a transgene, combined with the sequence of the antigen-binding region of the monoclonal anti-PrP antibody 6H4. This 6H4–IgM (6H4μ) was expressed on a $Prp^{+/-}$ background, and antibody titers were detectable. No autoimmune reactions or hematological disorders were observed. Only in Tg94 mice, which overexpress PrP^{c} on both B and T-cells, were 6H4μ-expressing B-cells strongly reduced.[151] $Prp^{+/-}$–6H4μ mice were i.p. inoculated, and prion accumulation in spleens and brains was monitored at different time points between 35 to 234 days dpi. In spleens 35 and 50 dpi, no infectivity was detectable in indicator bioassays, whereas the nontransgenic $Prp^{+/-}$ control group had developed measurable prion titers at these time points. In further analysis, spleens were tested by immunoblotting, and from both spleens and brains, histoblot analysis was performed after different time points. In $Prp^{+/-}$–6H4μ mice, no PrP^{Sc} accumulation was detected in any of the samples, a result indicating that both peripheral propagation and neuroinvasion were inhibited. No data are yet available about the incubation times to terminal prion disease in the $Prp^{+/-}$–6H4μ mice as compared to the control group. $Prp^{+/-}$ mice develop clinical prion disease between 400 and 465 dpi,[137] therefore the last time point analysed in this study (234 dpi) does not predict the further clinical development of the disease.

This seminal work in the field of antiprion immunisation found its justification in the more applied approach published recently by White et al.[154] This work showed that a traditional vaccination approach, namely passive application of anti-PrP antibodies, might indeed work against prion disease in vivo. These groups first generated mAbs in nontolerant $PrP^{-/-}$ mice by using recombinant murine PrP as an immunogen. By introducing either α-helical or β-sheet PrP they generated mAbs with different specificities towards PrP^{c} and PrP^{Sc}. The mAbs were then tested in a standard mouse model for prion infection. Mice were infected by the i.p. or i.c. route. mAbs were applied starting either from 7 or 30 dpi, with a continuous application twice a week at a dose of 2 mg (by using the i.p. route). As a control, non-PrP isotype mAbs were applied. At 60 dpi spleens were taken; PrP^{Sc} was determined by immunoblotting and prion infectivity was tested in indicator mouse bioassays. Both mAb treatments showed a dose- and time-dependent reduction of PrP^{Sc} and prion infectivity and a significant prolongation of incubation time when mice were infected by the i.p. route (>500 days when published). Interestingly, the best results were obtained with the mAb that was supposed to react mainly with PrP^{c}, a finding once again indicating that targeting PrP^{c} might be beneficial. Analysis of treated but healthy mice at 250 dpi showed that PrP^{Sc} was undetectable in the brain and reduced in the spleen. This gives hope that mAb treatment might result in a long-time effect, eventually even in complete protection. This was only the case when prions were applied by the i.p. route. Intracerebral inoculation was not at all influenced by mAb

treatment. This indicates that antibodies were not able to cross the blood – brain barrier at sufficient levels; this is different from the situation in Alzheimer's disease vaccination.[155, 156] As a note of caution for the prion field, vaccination approaches in the field of Alzheimer's disease revealed some unexpected autoimmunity side effects in the first clinical trials and showed that the transfer from experimental animal models to the situation in humans might be difficult.[157, 158]

Although active immunisation in prion disease is hampered by autotolerance against PrP,[137] it was shown that it is possible to induce autoantibodies in PrP-expressing mice,[159–162] and that autoantibodies have the potential to cure cells from prion infection.[162] Antibodies in PrP-expressing wild-type mice were induced either with full-length murine PrP that was chemically cross-linked with DnaK as an immunogen[159] or with PrP-derived peptides.[160] To test the functional effect of immunisation for the latter, the expression of PK-resistant PrP by a prion-infected tumour transplanted into immunised syngeneic A/J mice was examined. The induced immune response reduced the levels of PrP^{Sc} produced by the tumour without affecting the growth of the transplant. In another study, recombinant PrP in combination with Freund's adjuvant was used for immunisation of mice.[161] One group of animals was immunised before i.p. inoculation (prophylactic group), and a second group was immunised 24 h after inoculation (rescue group), with two dilutions of mouse prions. Antibody titers were measurable and the incubation time was prolonged in the prophylactic group with both dilutions of the inoculum, although the effect was more pronounced if the prions were more concentrated. This could be explained by higher antibody titers in these mice. Within the rescue group, no differences were observed in the tenfold dilution of the inoculum, whereas in the 1000-fold dilution, a slight difference in the incubation time was reported. Recently, an improved immunogen was introduced, namely a recombinantly expressed covalently linked PrP dimer consisting of a tandem duplication of two mouse PrP moieties (amino acids 23 – 231) linked by a seven amino acid linker.[162] Upon immunisation of mice, high antibody titers up to serum dilutions of 1:10 000 in ELISA were induced, and these polyclonal antibodies (pAbs) were tested for their ability to interfere with PrP^{Sc} biogenesis in ScN2a cells. This treatment induced the formation of insoluble full length PrP and inhibited the de novo synthesis of PrP^{Sc}. When pAbs induced with monomeric recombinant PrP were compared to dimer-induced antibodies, the latter showed a significantly higher potential to inhibit PrP^{Sc} synthesis, and in treatment of cells for up to seven days, only dimer-induced pAbs were able to cure cells from PrP^{Sc}. Fab fragments derived from pAbs did not induce insolubility of PrP^{c}, so it can be argued that this effect is caused by cross-linking of adjacent PrP molecules by the bivalent IgG. Furthermore, such Fabs had almost no effect on PrP^{Sc} propagation. When epitope mapping was performed, only autoantibodies reacted against an epitope near the C terminus, by covering two residues of the epitope mapped for the putative protein X binding site. This, in combination with important conformational epitopes which are not detectable in the epitope mapping performed, might explain the special potential of polyclonal autoantibodies induced by dimeric PrP. This capacity of the PrP dimer to induce effective autoantibodies could also be explained by presenting a structure similar to folding intermediates that might occur during the prion conversion process. In both studies, no side-effects or autoimmune reactions were reported.

A very recent article published by the group of Cashman reported the production of PrP^{Sc}-specific antibodies.[163] It was found that induction of β-sheet structures in recombinant PrP leads to an increased solvent accessibility of tyrosine residues, so antibodies were raised against the PrP repeat motif tyrosine-tyrosine-arginine, which is located in the structured C-terminal part of PrP. Under native conditions, these antibodies recognised PrP^{Sc}, but not PrP^{c}, in infected brain homogenates and also low levels of PrP^{Sc} in dendritic cells of lymph nodes taken from scrapie-infected sheep. Given the obviously high sensitivity and specifity, these antibodies might provide a powerfool tool for both treatment and diagnosis of prion diseases.

Taken together, the immune system or components thereof seem to be promising targets for intervention, at least in a postexposure scenario. Further investigation is necessary for the development of an active immunisation, but given the absence of severe side effects in mice and the induction of effective autoantibodies in first trials, it seems to be a realistic goal.

10. Summary and Outlook

In recent years, a variety of novel aspects in the molecular and cellular biogenesis and pathogenesis of prion proteins have been elucidated, shedding much more light on the still highly enigmatic cellular conversion process of PrP^{c} into pathogenic PrP^{Sc}/prions. The importance of these findings is not always clear and some data are still controversial. A reason for this might be the fact that prion infectivity and PrP-induced neurodegeneration could be distinct aspects of prion proteins. Understanding the nature of the infectious agent is likely to be a prerequisite for developing effective and causative antiprion strategies, useful in therapy, prophylaxis, and certain postexposure scenarios. The background for such means is highly interconnected with vCJD and its future development in case numbers. The question arises of whether one really wants to make use of such possibilities and whether this can be beneficial in human patients with advanced clinical symptoms. Nevertheless, a variety of putative antiprion strategies and compounds have been reported in different experimental model systems, including some interesting transgenic mouse models. The most surprising and unexpected developments come from the new field of antiprion immunisation. Passive and eventually even active immunisation approaches against prion infections are becoming realistic goals. Again, one has to ask whether results from mouse studies can be confirmed in higher mammalian species, most probably including primates, before vaccination studies in human prion disease situations can be performed. However, the perspective for real therapeutic or prophylactic intervention in human prion diseases is coming closer.

Abbreviations

BBB	blood – brain barrier
BSE	bovine spongiform encephalopathy
CFA	complete Freund's adjuvant
CJD	Creutzfeldt – Jakob disease
CLDs	caveolae-like domains
CNS	central nervous system
CsA	cyclosporin A
CWD	chronic wasting disease
DOSPA	2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate
dpi	days post infection
ER	endoplasmic reticulum
ERAD	ER-associated degradation
Fab	fragment antigen binding
FDC	follicular dendritic cell
FFI	fatal familiar insomnia
FSE	feline spongiform encephalopathy
GPI	glycosylphosphatidylinositol
GSS	Gerstmann – Sträussler – Scheinker syndrome
i.c.	intracerebral
Ig	immunoglobulin
i.p.	intraperitoneal
LR	laminin receptor
LRP	laminin receptor precursor
LT	lymphotoxin
mAb	monoclonal antibody
ODN	oligodeoxynucleotide
pAbs	polyclonal antibodies
PAMAM	polyamidoamide
PK	proteinase K
PPIases	peptidyl prolyl isomerases
PrP^{c}	cellular prion protein
PrPres	PK-resistant prion protein
PrP^{Sc}	pathological isoform of prion protein
TSE	transmissible spongiform encephalopathy
vCJD	(new) variant Creutzfeldt – Jakob disease
wt	wild-type

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